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Molecular form and concentration of serum α_2 -macroglobulin in diabetes

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α_2 -Macroglobulin is a highly abundant serum protein involved in the development of atherosclerosis and cardiac hypertrophy. However, its circulating molecular form and exact concentrations in human health/diseases are not known. Blue native-polyacrylamide gel electrophoresis of human serum was used to confirm the native conformation of α_2 -macroglobulin. We created an enzyme-linked immunosorbent assay suitable for quantifying its circulating molecular form and undertook a cross-sectional study to measure its serum levels in 248 patients with diabetes mellitus and 59 healthy volunteers. The predominant circulating molecular form of α_2 -macroglobulin was the tetramer, whereas its dimer was detectable in patients with high serum levels of α_2 -macroglobulin. The serum α_2 -macroglobulin concentration was not associated with glycated hemoglobin or any other glycemic variable as evaluated from 48-h continuous glucose monitoring, but showed close correlation with left ventricular posterior wall thickness, carotid artery intima-media thickness, urinary albumin:creatinine ratio (ACR) and brachial-ankle pulse wave velocity (baPWV). Multivariate analysis revealed only the ACR and baPWV to be independent variables influencing serum levels of α_2 -macroglobulin. Thus, an increased ACR and baPWV are associated with higher serum concentrations of α_2 -macroglobulin, and the latter may contribute to the mechanism by which albuminuria increases the risk of developing cardiovascular diseases.

In humans, α_2 -macroglobulin is the largest non-immunoglobulin molecule among the highly abundant proteins in the peripheral blood circulation. α_2 -macroglobulin is synthesized mainly in the liver as a result of coordination between endothelial cells and hepatocytes¹. α_2 -macroglobulin can inhibit a broad spectrum of serine, threonine, and metalloproteases as well as pro-inflammatory cytokines². It can also induce transcriptional activation of various genes essential for the proliferation/hypertrophy of cells, oncogenesis and atherosclerosis³.

Studies using stored pooled human plasma have suggested that two identical α_2 -macroglobulin subunits of size 182 kDa are disulfide-bonded to form dimers, which interact non-covalently to yield a tetrameric structure^{4,5}. In biologic fluids, α_2 -macroglobulin tetramers appear to be predominant, but may undergo structural changes during manipulation and preservation⁶. Thus, the exact circulating molecular forms of α_2 -macroglobulin associated with health and diseases have yet to be elucidated.

A 182-kDa protein termed “cardiac isoform of α_2 -macroglobulin” was shown to induce expression of muscle-specific genes associated with the pressure-overloaded heart and to cause cardiac hypertrophy directly^{7–10}. This putative “isoform” was claimed to be a key molecule inducing myocardial infarction and cardiac hypertrophy, especially in people with diabetes mellitus (DM) on the basis of serum level measurements^{11–13}. Recently, we and others demonstrated using mass spectrometry that the corresponding proteins in rats and humans were indistinguishable from α_2 -macroglobulin molecule^{9,14}.

Serum levels of α_2 -macroglobulin were determined first by Ganrot and Scherstén in 1967¹⁵ and have been shown to be increased in some DM populations, females and certain age groups^{15,16}. Increased levels in DM patients were later ascribed mostly to associated conditions, such as microvascular complications¹⁷, or worsened

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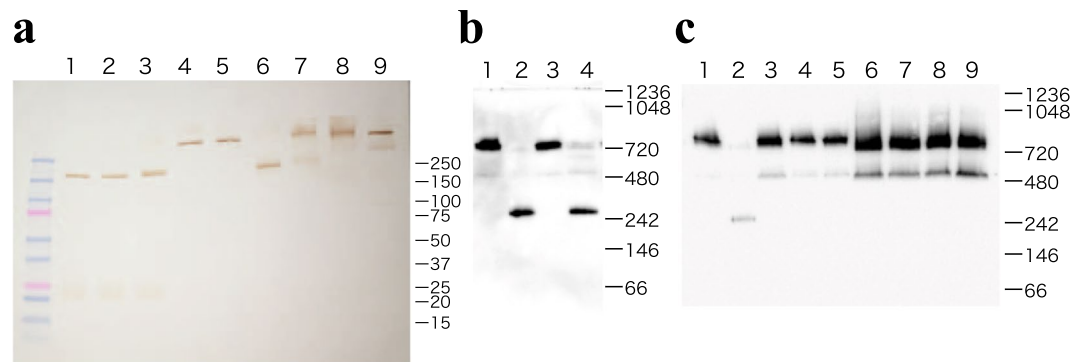


Figure 1. Molecular form of circulating α_2 -macroglobulin molecules in humans. **(a)** Representative SDS-PAGE under reducing and non-reducing conditions of purified human α_2 -macroglobulin protein and human serum. Ten-microliters of fresh human serum diluted to 1:80 and pretreated with 25 mM (lane 1), 10 mM (lane 2), 1 mM (lane 3), 0.1 mM (lane 4), or 0.01 mM of DTT (lane 5), 0.5 μ g of purified α_2 -macroglobulin protein pretreated with 25 mM (lane 6) or 0.1 mM of DTT (lane 7), and 10 μ L of fresh human serum diluted to 1:80 and pretreated with (lane 8) or without 0.5% SDS (lane 9) were subjected to 4–20% gradient gel electrophoresis and immunoblotted using human α_2 -macroglobulin antibody. **(b)** BN-PAGE of human serum and purified α_2 -macroglobulin protein pretreated with and without DTT. Purified α_2 -macroglobulin protein (0.1 μ g) pretreated without (lane 1) or with 25 mM of DTT (lane 2) and 10 μ L of fresh human serum diluted to 1:400 pretreated without (lane 3) or with 25 mM of DTT (lane 4) were subjected to BN-PAGE and subsequent immunoblotting using human α_2 -macroglobulin antibody. **(c)** BN-PAGE of purified α_2 -macroglobulin protein and diluted sera and subsequent immunoblotting using human α_2 -macroglobulin antibody. Purified α_2 -macroglobulin (0.05 μ g) protein pretreated without (lane 1) or with 25 mM of DTT (lane 2), and 10 μ L of diluted sera (1:400) obtained from 3 healthy volunteers (lane 3–5) and 4 patients with diabetes (lane 6–9) showing increased α_2 -macroglobulin concentrations were subjected to BN-PAGE and subsequent immunoblotting. The figure shows the cropped blots and the full-length blots are presented in Supplementary Fig. 1a–c.

glycemic control^{18–21}. However, those classical studies employed several assay methods using limited numbers of samples, whereas recent studies using commercial enzyme-linked immunosorbent assay (ELISA) kits have reported serum levels of α_2 -macroglobulin to be low^{8,11,12,22–25}. The concentrations reported thus far have been very inconsistent, ranging widely over six orders of magnitude^{15–23,25–27}. Also, the pathophysiologic role of this factor in human diseases remains unknown.

Microalbuminuria has been measured to assess renal risk in early diabetic nephropathy, but is also a strong predictor of cardiovascular diseases^{28–30}. People with type-1 diabetes mellitus (T1DM) and nephropathy carry a tenfold greater risk of adverse cardiovascular outcome compared with those without these disorders³¹. A slightly increased urinary albumin:creatinine ratio (ACR) carries an increased atherosclerotic risk³², which is reduced by the regression of albuminuria^{28,33}. The latter is also associated with premature death^{34–37} which, in those with T1DM, is due mainly to cardiovascular factors³⁷. However, the mechanisms relating albuminuria with an increased cardiovascular risk are not known.

We established a new ELISA to quantify circulating levels of the molecular form of human α_2 -macroglobulin using an antibody that can recognize its monomers, dimers and tetramers in human serum. We designed a cross-sectional study to ascertain if this important molecule is related to diabetic microvascular complications and the risk factors of cardiac/atherosclerotic diseases.

Results

To study the native conformation of circulating α_2 -macroglobulin molecules, we incubated purified α_2 -macroglobulin protein and fresh human serum treated with and without dithiothreitol (DTT) and subjected them to sodium dodecyl sulfate (SDS)-PAGE and western blotting. A single band representing α_2 -macroglobulin-like immunoreactivity corresponding to the size of standard α_2 -macroglobulin protein was detected on western blots from healthy human serum. Treatment of serum and an α_2 -macroglobulin standard with increasing concentrations of DTT (1, 10 and 25 mM) reduced the standard α_2 -macroglobulin protein to form monomers of size 182 kDa (Fig. 1a). Blue native (BN)-PAGE of serum and standard α_2 -macroglobulin protein and subsequent immunoblotting revealed a single band corresponding to an α_2 -macroglobulin tetramer of size 725 kDa in untreated samples, whereas treatment of serum and standard α_2 -macroglobulin with DTT reduced the protein into monomers of size 182 kDa (Fig. 1b). A band corresponding to an α_2 -macroglobulin dimer was very faint or barely visible in the lane for a sample of healthy serum or in the lane for standard tetrameric α_2 -macroglobulin. However, in some untreated sera of patients with DM who showed increased serum levels of α_2 -macroglobulin, dimers of α_2 -macroglobulin were visible (Fig. 1c). These results suggested that circulating α_2 -macroglobulin molecules in healthy humans consisted of tetramers that could be monomerized completely by reducing intermolecular disulfide bonds, and that dimers could be present in some patients with DM.

We established an ELISA using anti-human monoclonal mouse α_2 -macroglobulin IgG, which has been confirmed to recognize monomeric, dimeric and tetrameric α_2 -macroglobulin molecules. A curve prepared with tetrameric α_2 -macroglobulin standards (0.0–0.2 μ g/mL) showed parallelism with those of serially diluted serum

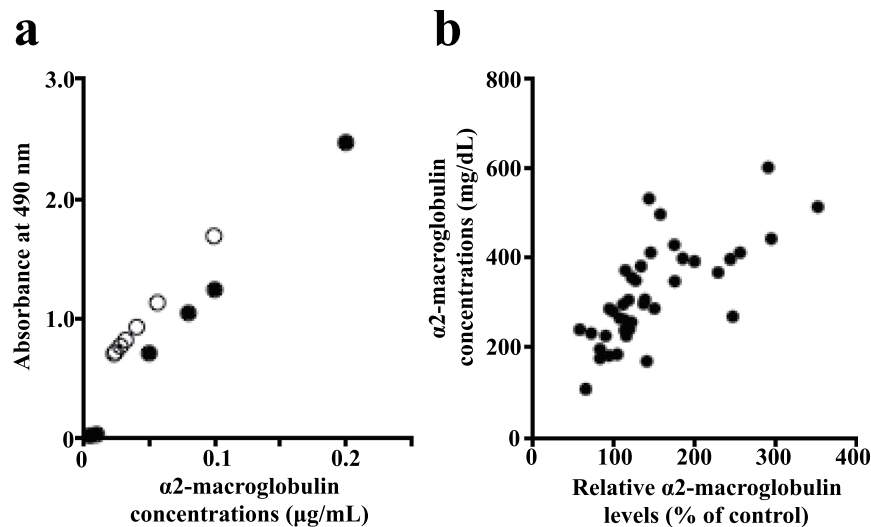


Figure 2. α_2 -macroglobulin ELISA. (a) Parallelism of the dilution curve generated by normal human serum serially diluted to 1:40000, 1:80000, 1:120000, 1:160000, 1:200000, 1:240000, and 1:320000 (open circles) with the human α_2 -macroglobulin standard regression curve (closed circles). (b) Serum α_2 -macroglobulin levels of 40 DM patients were determined using quantitative western blotting and by ELISA, and the two values were plotted to observe potential correlations.

	Healthy volunteers	Diabetes mellitus	p
Number (male/female)	59 (26/33)	248 (133/115)	
Diabetes type (type1/2/gestational)		39/204/5	
Age (years)	54.3 \pm 13.2	58.3 \pm 15.9	0.0747
Body mass index (kg/m ²)	22.8 \pm 3.4	25.5 \pm 5.0	0.0010
HbA _{1c} (%)	5.5 \pm 0.3	9.8 \pm 2.7	<0.0001
Serum creatinine (mg/dL)	0.71 \pm 0.16	0.85 \pm 0.42	0.0668
LDL-Cholesterol (mg/dL)	117.3 \pm 38.3	115.2 \pm 37.0	0.7787
HDL-Cholesterol (mg/dL)	69.1 \pm 16.5	51.7 \pm 16.0	<0.0001
Triglyceride (mg/dL)	115.1 \pm 58.4	162.9 \pm 133.5	0.0539
Retinopathy (none/simple/prepro/pro)		180/36/24/8	
Albuminuria categories (+1/+2/+3)		131/74/43	

Table 1. Characteristics of study participants. HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA_{1c}, glycated hemoglobin; +1: albumin:creatinine ratio <30 mg/gCr, +2: 30–299 mg/gCr, +3: >300 mg/gCr.

samples in the ELISA of α_2 -macroglobulin (Fig. 2a). When serial dilutions of serum were applied to the standard curve, a very good correlation was obtained ($R^2 = 0.9646$). The concentration of unknown samples was determined by interpolation, which is reliant on a standard curve being generated appropriately. The intra-assay coefficient of variation (CV) of serum samples was 2.3% ($n = 8$) and inter-assay CV was 13.8% ($n = 14$). These results suggested that our ELISA was suitable for quantifying serum concentrations of α_2 -macroglobulin in humans. Using serum samples from 40 DM patients, we compared the results obtained with the current ELISA and those determined using quantitative western blotting¹⁴ (in which α_2 -macroglobulin molecules in serum should undergo reduction/monomerization during SDS-PAGE). Data for the ELISA of α_2 -macroglobulin showed close correlation with the total concentration of monomerized α_2 -macroglobulin ($R^2 = 0.575$, $p < 0.0001$) (Fig. 2b).

We measured serum concentrations of α_2 -macroglobulin in 59 healthy volunteers and 248 patients with DM (Table 1). Patients with DM showed significantly higher serum levels of α_2 -macroglobulin (310.4 ± 114.2 mg/dL) than healthy volunteers (272.9 ± 76.5 mg/dL) ($p = 0.0169$) (Fig. 3). When the data of all 307 subjects were analyzed together, serum levels of α_2 -macroglobulin did not correlate with HbA_{1c} level. To confirm no association between serum α_2 -macroglobulin with glycaemic control or glycaemic fluctuation in DM, we analyzed the results of 57 individuals who underwent 48-h CGM at the time of measuring serum levels of α_2 -macroglobulin. Neither the mean glucose level nor data on glycemic fluctuation (standard deviation, CV, mean amplitude of glycemic excursions) showed a correlation with serum levels of α_2 -macroglobulin. These results negate the current belief that serum concentrations of α_2 -macroglobulin reflect glycemic control.

Pearson's univariate correlation analysis revealed a positive correlation of serum levels of α_2 -macroglobulin with the ACR, baPWV, maximum intima media thickness (maxIMT), left ventricular posterior wall thickness

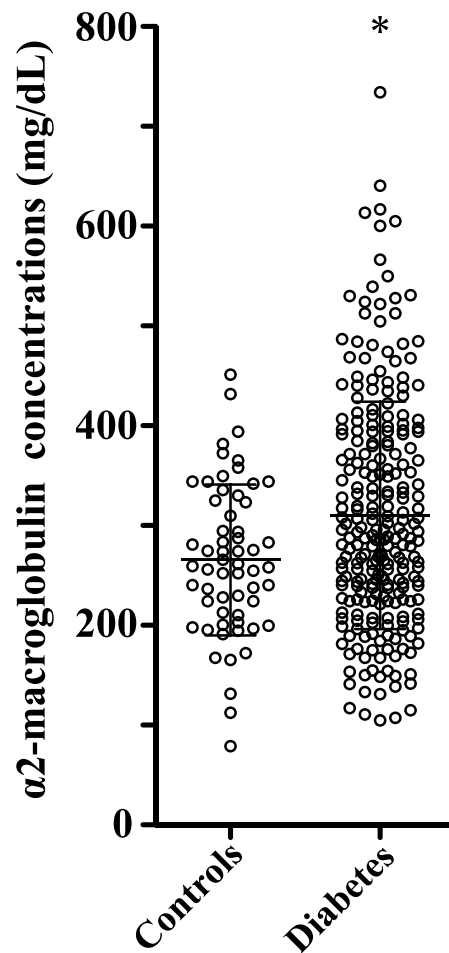


Figure 3. Comparison of serum α_2 -macroglobulin levels in healthy volunteers and in patients with diabetes mellitus. Serum samples obtained from 59 healthy volunteers (Controls) and 248 patients with diabetes were subjected to ELISA of α_2 -macroglobulin. Wide horizontal bars indicate the mean and the shorter horizontal bars indicate the SD. The unpaired *t*-test was used to compare serum levels for the two groups.**p* < 0.05 vs. controls.

Parameter	r	P
Age (years)	0.183	0.0013
Male: female		0.0322
BMI (kg/m ²)	0.0790	0.1812
HbA _{1c} (%)	-0.0185	0.7616
Retinopathy +/-		0.0020
ACR (mg/gCr)	0.236	0.0002
eGFR (mL/min/1.73 m ²)	-0.136	0.0226
baPWV (cm/s)	0.233	0.0005
maxIMT (mm)	0.279	<0.0001
LVPWth (mm)	0.272	0.0039
IVSth (mm)	0.230	0.0139

Table 2. Correlation between patient parameters and serum level of α_2 -macroglobulin: univariate analysis. BMI = body mass index, ACR = albumin:creatinine ratio, eGFR = estimated glomerular filtration rate, baPWV = brachial-ankle pulse wave velocity, maxIMT = maximal intima-media thickness. LVPWth = left ventricular posterior wall thickness, IVSth = interventricular septum thickness.

(LVPWth) and interventricular septum thickness (IVSth), but not with HbA_{1c} levels (Table 2). Serum levels of α_2 -macroglobulin in DM patients with retinopathy were higher than those without (Table 2). Of 248 patients with diabetes, 51 had cardiovascular disease as evidenced by coronary angiography, CT of coronary arteries

Parameters	β	F	P
Age (years)	0.0929	0.9871	0.3217
Male: female	0.1002	2.0381	0.1550
Retinopathy +/-	-0.0028	0.0014	0.9705
ACR (mg/g)	0.2033	7.4355	0.0070
eGFR (mL/min/1.73 m ²)	0.0510	0.3621	0.5480
baPWV (cm/sec)	0.1757	4.1495	0.0430
Cardiovascular disease +/-	0.0864	1.3559	0.2457

Table 3. Correlation between characteristics of DM patients and serum level of α_2 -macroglobulin: least square multivariate analysis. ACR = albumin creatinine ratio, eGFR = estimated glomerular filtration rate, baPWV = brachial-ankle pulse wave velocity.

and/or cardiac ultrasonography. Least square multivariate analyses revealed that only the ACR and baPWV were associated independently with higher serum levels of α_2 -macroglobulin, and that age, sex, eGFR or the presence of retinopathy or cardiovascular disease were not independent variables influencing serum levels of α_2 -macroglobulin (Table 3). Also, serum levels of α_2 -macroglobulin in DM patients with normoalbuminuria (281.0 ± 95.1 mg/dL, $n = 131$) were not significantly higher than those of healthy volunteers (272.9 ± 76.5 mg/dL, $n = 59$). Taken together, these results suggested that the ACR and baPWV had an impact on higher serum concentrations of α_2 -macroglobulin.

Discussion

We developed a highly sensitive ELISA to recognize human α_2 -macroglobulin molecules in serum specifically. The limit of detection was 0.5 ng/mL, and the standard curves obtained by diluting the serum from 1:40,000 to 1:320,000 showed parallelism with the standard purified tetramer protein of α_2 -macroglobulin. Inter-assay and intra-assay CVs were small, and the results were stably reproducible. α_2 -macroglobulin levels in human serum have ranged by six orders of magnitude, and reported to be 0.5–2.5 μ g/dL when measured using commercial ELISA kits^{22,23,25}. Albumin and Ig molecules make up the greatest proportion of plasma proteins, but α_2 -macroglobulin is regarded to be one of top-12 abundant proteins. In the present study, the serum concentration of α_2 -macroglobulin in healthy volunteers was 272.9 ± 76.5 mg/dL, which was quite reasonable.

Considering the well-described potent biologic activities of α_2 -macroglobulin^{10,38–41} and its predominance over other bioactive substances in plasma, significant fluctuations of this important molecule in the peripheral blood circulation could affect the onset and/or progression of human diseases. Our system is the first ELISA suitable for quantifying α_2 -macroglobulin levels in human serum accurately.

BN-PAGE revealed that the α_2 -macroglobulin-like immunoreactivity in human serum migrated to a position corresponding to that of the purified tetramer protein of α_2 -macroglobulin of size 725 kDa. Reduction of the disulfide bonds of purified α_2 -macroglobulin protein and α_2 -macroglobulin molecules in human serum by DTT treatment followed by BN-PAGE and western blotting revealed that α_2 -macroglobulin molecules in serum were separated completely into monomers of size 182 kDa even in the absence of SDS. Furthermore, neither serum nor purified α_2 -macroglobulin protein were monomerized following SDS-PAGE due to non-covalent bonding, whereas both were monomerized completely with reduction of disulfide bonds after DTT treatment. These data argue against the current belief based on studies using stored human plasma that two disulfide-bonded α_2 -macroglobulin dimers are non-covalently bonded to form tetramers in the peripheral blood circulation^{2,4,5,42}.

In some patients with DM whose serum α_2 -macroglobulin levels were increased markedly, dimeric components were observed in addition to tetramers according to BN-PAGE. Thus, dimers of α_2 -macroglobulin may be formed in human blood if α_2 -macroglobulin synthesis is enhanced. Our results demonstrate that the α_2 -macroglobulin antibody that we used had cross-reactivity with monomers, dimers and tetramers, which could be present in human serum. We compared the results of semiquantitative measurement of serum α_2 -macroglobulin monomerized after DTT treatment and SDS-PAGE with measurements by ELISA using 40 samples of human serum, and found that both were highly correlated (Fig. 2b). We concluded that our ELISA reflected the circulating components of human α_2 -macroglobulin molecules.

We also found significantly high serum levels of α_2 -macroglobulin in DM patients compared with healthy volunteers. Several studies have reported a positive correlation between the HbA_{1c} level and α_2 -macroglobulin level in serum^{20–22}, but we did not. To negate the possibility that blood glucose levels in DM affect serum concentrations of α_2 -macroglobulin, we obtained the exact glycemic profiles of 57 individuals through 48-h CGM. Neither the mean blood glucose levels of the entire 48-h recordings nor variables of glycemic fluctuation (standard deviation, CV) correlated with serum levels of α_2 -macroglobulin. Univariate regression analysis of 248 DM patients revealed that the α_2 -macroglobulin level correlated with the ACR, baPWV, maxIMT, LVPWth, IVSth, but not with the HbA_{1c} level. Serum levels of α_2 -macroglobulin were significantly higher in DM patients with retinopathy than those without retinopathy. Among these variables, however, multiple regression analysis with the α_2 -macroglobulin level as the objective variable revealed only the ACR and baPWV to be significant independent variables affecting the serum level of α_2 -macroglobulin. The association of the urinary albumin concentration with an increased level of this important molecule may be linked to the mechanism by which albuminuria increases the risk of developing cardiac and atherosclerotic diseases.

Albuminuria was originally identified as an incipient marker of diabetic nephropathy^{43,44}, but is now well documented as an independent predictor of cardiovascular morbidity and mortality not only in T1DM and

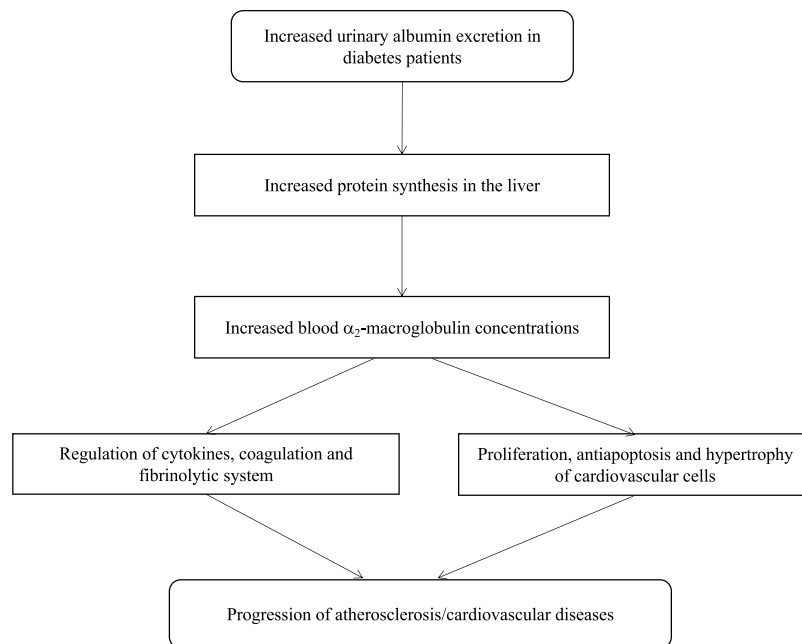


Figure 4. Flowchart for the proposed mechanistic link between albuminuria, serum α_2 -macroglobulin and cardiovascular disease in diabetes.

T2DM^{28,37,45,46}, but across varied non-DM populations^{29,36,47,48}. Accumulating evidence suggests that proteinuria is a stronger predictor of cardiovascular disease than traditional risk factors such as cholesterol and blood pressure^{30,49}, whereas albuminuria is associated with an increased risk starting well below the cutoff for microalbuminuria^{29,48,49}. However, the exact pathophysiologic conditions responsible for the overwhelming cardiovascular consequences induced specifically by trace albuminuria, but not in patients without albuminuria, have remained unknown to date.

Several mechanisms have been proposed to contribute to the cardiovascular consequences of albuminuria, including inflammation, endothelial dysfunction, thrombogenic factors, and insulin resistance^{50–53}. In nephrotic syndrome, hepatic synthesis of the high-molecular-weight protein α_2 -macroglobulin has been shown to be enhanced significantly to replace lost liver-derived proteins in experimental animals⁵⁴ and humans⁵⁵, resulting in a net increase in its serum levels⁵⁴. The present study demonstrated a close correlation between serum levels of α_2 -macroglobulin and the ACR, and that the former start to rise when a trace amount of albumin is excreted. Considering the magnitude of increased post-transcriptional α_2 -macroglobulin synthesis in the liver in response to urinary protein excretion^{54–56}, it is reasonable to assume that circulating α_2 -macroglobulin levels may be increased far more profoundly by urinary albumin than by other factors such as sex, age, glycemic control or diabetic retinopathy. A proposed mechanistic link relating albuminuria to increased serum level of α_2 -macroglobulin and cardiovascular disease in diabetes is depicted in Fig. 4.

Considering the limited number of individuals employed in this cross-sectional study, whether an increased serum level of α_2 -macroglobulin in diabetes mellitus could serve as a mediator of albuminuria that, ultimately, results in cardiovascular diseases, is not known. Also, whether preventive therapy can reverse albuminuria and reduce the serum level of α_2 -macroglobulin is not known. Further studies to ascertain if measurement of the serum level of α_2 -macroglobulin could help to identify patients at a high risk of cardiovascular disease should be carried out.

In conclusion, using a sensitive and specific ELISA, we identified, among other laboratory parameters, a close association between serum levels of α_2 -macroglobulin with ACR and baPWV. Measurement of serum levels of α_2 -macroglobulin could help to identify patients at high risk of cardiovascular diseases.

Methods

Study participants. We enrolled 248 patients with DM (39 with T1DM, 204 with T2DM, and 5 with gestational DM) and 59 healthy volunteers at Kitasato University Medical Center or Kitasato University Hospital. Excluded from the analyses were patients with infectious diseases, malignancy, end-stage renal failure, autoimmune thyroid diseases and those receiving corticosteroids. We obtained information on the medical and life histories of all participants.

Patients underwent routine evaluation of systemic diseases covered by the universal health coverage system in Japan^{57,58}. These included electrocardiography (ECG), radiography (chest and abdomen), ultrasonography (neck and/or abdomen), urinalysis, complete blood count, biochemical analyses of serum, as well as tests for levels of free thyroxine, free triiodothyronine, thyroid-stimulating hormone (TSH) and thyroid antibodies. DM patients underwent ophthalmologic and neurologic tests, the anti-glutamic acid decarboxylase antibody test as well as measurements of glycated albumin and/or glycated hemoglobin (HbA_{1c}), fasting serum insulin, and urinary ACR.

Glycemic control/fluctuation was assessed in 54 DM patients without macroalbuminuria (18 males and 36 females; 41.3 ± 13.5 years; HbA_{1c} , $8.0 \pm 1.8\%$) and 3 people without DM (3 females; 40.0 ± 2.6 years; $5.7 \pm 0.2\%$) using 48-h continuous glucose monitoring (CGM) recording (CGMS[®] GOLD, Medtronic Minimed, Northridge, CA, USA)^{59,60}.

Cardiovascular diseases were diagnosed by ECG, exercise electrocardiography, ankle-brachial pressure index, brachial-ankle pulse wave velocity (baPWV), cardiac catheterization/angiography, and/or coronary computed tomography. For healthy volunteers, the blood-test data at the most recent medical checkup were used to confirm the absence of other systemic diseases and as analytical data.

Collection of serum samples. Blood samples from study participants were collected into vacutainers. Serum was separated immediately in a refrigerated centrifuge and stored in aliquots at -30°C until processing.

Materials. Purified human α_2 -macroglobulin protein (725 kDa) was obtained from Enzo Life Science (New York, USA), anti-human monoclonal mouse α_2 -macroglobulin (immunoglobulin (Ig)G1 clone) from R&D Systems (Minneapolis, MN, USA), and goat anti-mouse IgG (H + L)-horseradish peroxidase (HRP) conjugate from Bio-Rad Laboratories (Hercules, CA, USA). Native PAGE[™] Sample Buffer, Native PAGE Running Buffer, Dark Blue Cathode Buffer, Native Mark[™] Unstained Protein Standard, and Light Blue Cathode Buffer were obtained from Thermo Fisher Scientific (Waltham, MA, USA). DTT, Blocking One and Peroxidase Stain 3,3'-Diaminobenzidine kit (Brown Stain) were purchased from Nacalai Tesque (Kyoto, Japan). Immune-Blot[®] polyvinylidene difluoride membrane, Sequi-Blot[™] membrane, Precision Plus Protein[™] Dual Color Standards and Mini-PROTEAN[®] TGX[™] were obtained from Bio-Rad Laboratories. Perfect NT Gel, Perfect NT Gel System and SDS-PAGE Running Buffer were obtained from DRC (Tokyo, Japan), and ECL Prime Western Blotting Detection Reagent from GE Healthcare (Buckinghamshire, UK).

ELISA of α_2 -macroglobulin. All measurements were carried out in triplicate. One-hundred microliters of human α_2 -macroglobulin protein diluted to 0.5–80 ng/mL and serum samples diluted to 1:160,000 with phosphate-buffered saline (PBS) were incubated overnight at 4°C in a 96-well microplate. Plates were washed thrice and blocked with 200 μL /well of PBS containing 0.05% Tween[®]20 (PBS-T) and 5% (*w/v*) skimmed milk/Tris-buffered saline (TBS) for 2 h at room temperature. After washing thrice with PBS-T (200 μL /well), plates were incubated overnight with 100 μL of anti-human monoclonal mouse α_2 -macroglobulin at 1:5,000 dilution. Then, plates were washed thrice with PBS-T (200 μL /well) and reacted with 1:10,000 goat anti-mouse IgG (H + L)-HRP conjugate (100 μL /well) and allowed to stand at room temperature for 1 h under light-shielding. After washing four times with PBS-T (200 μL /well), *o*-phenylenediamine/0.05 M phosphate-citrate buffer (100 μL /well) was added for 30 min at room temperature under light-shielding, then overlaid with 3 N HCl (100 μL /well) to terminate the reaction. Absorbance of the plate was measured at 490 nm using a microplate reader (iMark[™]; Bio-Rad Laboratories).

Polyacrylamide gel electrophoresis (PAGE) followed by western blotting. SDS-PAGE was performed using purified human α_2 -macroglobulin protein and human serum essentially as described^{61,62} except for the following modifications. Ten-microliters of fresh human serum diluted to 1:80 and 0.5 μg of purified α_2 -macroglobulin protein pretreated with or without DTT were subjected to 4–20% gradient gel electrophoresis using Mini-PROTEAN[®] TGX[™] and subsequent immunoblotting using human α_2 -macroglobulin antibody.

Blue native PAGE was performed as described⁶³ except for the following modifications. Native PAGE[™] Sample Buffer was used to dissolve 0.1 μg of standard α_2 -macroglobulin and to dilute 0.025 μL of serum. After pretreatment with or without 25 mM of DTT, 10 μL of diluted samples was loaded on a 5–20% gradient Perfect NT Gel using Native PAGE Running Buffer as a positive electrode and Dark Blue Cathode Buffer as a negative electrode according to manufacturer instructions. Native Mark[™] Unstained Protein Standard was used as a marker of molecular size. After migrating samples at 150 V to one-third of the entire gel, Dark Blue Cathode Buffer in the negative electrode was replaced with Light Blue Cathode Buffer to complete the remaining electrophoresis.

After electrophoresis, the gel was photographed, transferred to a PVDF membrane (Immune-Blot[®]) at 75 V for 120 min, incubated with 8% acetic acid for 15 min, then with sterilized water for 5 min, and air-dried to fix the protein. The PVDF membrane was then immersed in MeOH to remove Coomassie brilliant blue G-250 dye. Then, it was washed with sterilized water, blocked at 4°C overnight with Blocking One and, after washing thrice with TBS-T, incubated overnight with human α_2 -macroglobulin antibody (1:5000 dilution) at 4°C . After incubation with goat anti-mouse IgG (H + L)-HRP Conjugate (1:10000 dilution) for 1 h, protein bands were detected using ECL and photographed and analyzed with ImageQuant LAS 4000 (GE Healthcare).

Quantitative western blotting to measure levels of monomerized α_2 -macroglobulin. Serum samples from 40 consecutive DM patients were diluted 40-fold with binding buffer (50 mM DTT, 0.5% SDS, 10% glycerol/1 M Tris-HCl) and subjected to SDS-PAGE with a protein marker (Precision Plus Protein[™] Dual Color Standards) using a 5% Perfect NT Gel and a Perfect NT Gel System SDS-PAGE Running Buffer at 120 V for 60–90 min. Proteins in the gels were then transferred to a PVDF membrane (Sequi-Blot[™]) at 60 mA for 60 min and incubated overnight at 4°C with Blocking One. After washing thrice with TBS-T, the PVDF membrane was incubated with human α_2 -macroglobulin antibody (1:3000 dilution) for 90 min, and then with goat anti-mouse IgG(H + L)-HRP Conjugate (1:5000 dilution) for 1 h at room temperature. The PVDF membrane was visualized using Brown Stain. The intensity of each band was quantified using the ImageQuant TL program (GE Healthcare). All densitometric data were converted to relative monomer concentrations using twofold serially diluted samples of control protein.

Statistical analyses. Data are the mean \pm SD unless indicated otherwise. Statistical analyses were carried out using JMP v5 (SAS institute, Cary, NC, USA) and Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between groups were examined for significance using the unpaired *t*-test. Correlation analysis was

undertaken on the relationship between the α_2 -macroglobulin level and each parameter. Multiple regression analyses were done employing age, sex, diabetic retinopathy, the ACR, estimated glomerular filtration rate (eGFR), baPWV and cardiovascular disease as an explanatory variable and serum level of α_2 -macroglobulin as an objective variable. $P < 0.05$ was considered significant.

Ethics approval and consent to participate. The study protocol was approved by the Ethics Committees of Kitasato University Medical Center (Saitama, Japan; 29–27) and Kitasato University Hospital (Kanagawa, Japan; B17-119). All study methods were undertaken in accordance with the relevant guidelines and regulations of these two organizations as well as the *Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan*. Written informed consent was obtained from all participants.

Data Availability

All data generated or analyzed during this study are included in this article.

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Author Contributions

S.Y. and K.F. processed serum samples, established the ELISA and measured α_2 -macroglobulin levels in serum. T.T. and J.O. collected serum samples and clinical information on DM patients. Y. Ka. and M.S. collected serum samples from patients with DM, evaluated their clinical course and confirmed the final diagnoses. S.Y., K.F., S.K. and Y. Ko. undertook native gel electrophoresis and confirmed circulating levels of α_2 -macroglobulin. S.Y., J.O. and T.T. undertook statistical analyses and M.S. confirmed the results. M.S. conceived experiments, analyzed data and secured funding. M.S. wrote, reviewed and edited the manuscript. All authors discussed the results and commented on the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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