

# Urinary Angiotensinogen in Patients With Type 1 Diabetes With Microalbuminuria: Gender Differences and Effect of Intensive Insulin Therapy



Jessica Navarro<sup>1</sup>, Alejandro Sanchez<sup>1</sup>, Sheeba H. Ba Aqeel<sup>1</sup>, Minghao Ye<sup>1</sup>, Mohammed Z. Rehman<sup>1</sup>, Jan Wysocki<sup>1</sup>, Alfred Rademaker<sup>2</sup>, Mark E. Molitch<sup>3</sup> and Daniel Batlle<sup>1</sup>

<sup>1</sup>Division of Nephrology and Hypertension, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA; <sup>2</sup>Division of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA; and <sup>3</sup>Division of Endocrinology, Metabolism and Molecular Medicine, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

**Introduction:** Angiotensinogen (AOG) is the precursor of peptides of the renin angiotensin system (RAS). Because insulin up-regulates transcriptional factors that normally repress kidney AOG synthesis, we evaluated urinary AOG (uAOG) in patients with type 1 diabetes (T1D) and microalbuminuria who are receiving either intensive or conventional insulin therapy.

**Methods:** Urine samples from participants of the Diabetes Control and Complications Trial (DCCT) were used for the following: (i) uAOG/creatinine measurements in 103 patients with microalbuminuria and 103 patients with normoalbuminuria, matched for age, gender, disease duration, and allocation to insulin therapy; and (ii) uAOG/creatinine measurements from patients with microalbuminuria allocated to intensive insulin therapy ( $n = 58$ ) or conventional insulin therapy ( $n = 41$ ) after 3 years on each modality.

**Results:** uAOG was higher in patients who started with microalbuminuria than in those with normoalbuminuria (6.65 vs. 4.0 ng/mg creatinine,  $P < 0.01$ ). uAOG was higher in females than in males with microalbuminuria (11.7 vs. 5.4 ng/mg creatinine,  $P = 0.015$ ). uAOG was lower in patients with microalbuminuria allocated to intensive insulin therapy than in conventional insulin therapy (3.98 vs. 7.42 ng/mg creatinine,  $P < 0.01$ ). These differences in uAOG were observed though albumin excretion rate (AER) was not significantly different.

**Conclusion:** In patients with T1D and microalbuminuria, uAOG is increased and varies with gender and the type of insulin therapy independently of AER. This suggests that AOG production is increased in females and it is decreased by intensive insulin therapy. The reduction in uAOG with intensive insulin therapy, by kidney RAS downregulation, may contribute to the known renoprotective action associated with intensive insulin and improved glycemic control.

*Kidney Int Rep* (2022) 7, 2657–2667; <https://doi.org/10.1016/j.ekir.2022.09.010>

KEYWORDS: albuminuria; angiotensinogen; renin-angiotensin system; diabetic kidney disease; Type 1 diabetes

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The RAS is a major regulatory pathway and an important therapeutic target to slow down the progression of chronic kidney disease.<sup>1–11</sup> AOG is the main parent compound for the formation of angiotensin peptides. Consistent with the important role of the RAS in hypertension and kidney disease, a recent analysis of

more than 200 genes for kidney function and hypertension found that AOG in proximal tubule cells emerged among the genes for complex kidney disease-associated phenotypes.<sup>12</sup> The main source of AOG is the liver, which produces it constantly to sustain a high level in plasma<sup>13</sup> but it is also produced in kidney proximal tubule cells.<sup>14–17</sup> AOG, like albumin, can be recovered in the urine in small amounts in healthy subjects because both proteins can pass a normal glomerular filtration barrier.<sup>16,18</sup> With diabetic kidney disease (DKD), uAOG levels are increased as reported from cross sectional studies that included patients with chronic kidney disease and associated

**Correspondence:** Daniel Batlle, Division of Nephrology and Hypertension, Department of Medicine, The Feinberg School of Medicine, Northwestern University, 320 E Superior, Chicago, Illinois 60611, USA. E-mail: [d-batlle@northwestern.edu](mailto:d-batlle@northwestern.edu)

Received 20 July 2022; revised 25 August 2022; accepted 12 September 2022; published online 20 September 2022

hypertension.<sup>19–23</sup> These patients, however, were treated with medications, including RAS blockers.<sup>19–23</sup> RAS blockers can decrease uAOG<sup>24–27</sup> and hypertension has been reported to increase urinary levels of AOG.<sup>28,29</sup> The foregoing indicates the need for studies where uAOG can be evaluated in patients with diabetes without hypertension and in the absence of other confounding factors, such as the use of RAS blockers.

uAOG in DKD is increased most likely as a result of augmented passage of liver derived AOG through an altered glomerular filtration barrier. Part of the AOG found in the urine, however, also originates in the proximal tubule, particularly if the synthesis at this site is increased.<sup>14–17</sup> This may be the case in DKD, because it is known that high glucose increases AOG mRNA in kidney tubular cells<sup>30–32</sup> and intrarenal AOG is increased in rodent models of T1D.<sup>30,33</sup> Gene transcription of AOG has been studied in cultured renal proximal tubular cells and in the Akita mouse model of T1D.<sup>30,31,33–36</sup> The best studied regulator of kidney AOG synthesis is insulin, which up-regulates 2 transcriptional factors, namely heterogeneous nuclear ribonucleoprotein F and K (hnRNP F and hnRNP K) that normally repress kidney AOG synthesis.<sup>33</sup> In rodent models of T1D, intrarenal (proximal tubule) AOG is increased, and this is attributable to suppression of hnRNP F as a result of insulin deficiency.<sup>30,37,38</sup>

In humans, the effect of insulin on uAOG has not been studied. In this report, AOG was determined in National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Repository urine samples from patients with T1D and microalbuminuria from participants in the DCCT. A unique feature of this population is that when the urine samples were obtained, study participants were not overtly hypertensive or receiving RAS blockers which could affect AOG. Because patients were allocated to either intensive insulin or conventional insulin for several years, we were able to examine the long-term effect of insulin on uAOG after at least 3 years on each therapeutic modality.

## METHODS

The study was based on deidentified historical samples, and it was considered exempt by the Northwestern University Institutional Review Board. Urine samples from participants in the DCCT were provided by the NIDDK repository. The DCCT was a multicenter randomized clinical trial, including 1441 volunteers (96%–97% Caucasian), aged 13 years to 39 years, with T1D of 1 year to 15 years duration from 29 medical centers in the United States and Canada conducted from 1983 to 1993, and aimed to compare conventional versus

intensive therapy on blood glucose control and complications of diabetes.<sup>39</sup>

The major inclusion criteria were insulin dependence (deficient C-peptide secretion); aged 13 years to 39 years; as well as absence of hypertension, hypercholesterolemia, and severe diabetic complications or medical conditions.<sup>40</sup> The exclusion criteria were duration since diagnosis less than 1 year or more than 15 years before enrollment, type 2 diabetes, history of cardiovascular disease, hypertension (BP  $\geq 140/90$  mm Hg), hyperlipidemia, serum creatinine  $\geq 1.2$  mg/dl or creatinine clearance  $\leq 100$  ml/min per  $1.73$  m<sup>2</sup> body surface area, severe diabetic complications, severe medical comorbidities.<sup>40</sup>

## Study Design

We requested from the NIDDK repository all the available urine samples from study participants in whom microalbuminuria was documented at the initiation of the DCCT. Out of all the 104 subjects with microalbuminuria at study initiation (cases) we were able to use samples from 103 who started the study with microalbuminuria, (defined as 24-hour albumin excretion between 30 and 300 mg/24h, [Supplementary Figure S1](#)). None of the study participants were taking RAS blockers throughout the DCCT. The AER in most cases was in the low microalbuminuric range (median 38.9 mg/24h). In normoalbuminuric controls, the median AER was 10.1 mg/24h. The characteristics of the 103 cases with microalbuminuria and controls with normoalbuminuria who were matched (as per our request to the NIDDK) for age, gender, disease duration and allocation to treatment group are shown in [Supplementary Table S1](#). Because samples for AOG measurements were not available at the initial entry visit we used the sample available from the earliest annual visit following study initiation (on average, year 2, range 0–8 see [Table 1](#)). At this study visit, however, 33 of the 103 individuals had AER values in the normoalbuminuric range whereas the remaining 70 had persistent microalbuminuria ([Figure 1a](#) and [1c](#)). Accordingly, we analyzed the data for all cases combined ( $N = 103$ ) ([Table 1](#)) and in a subset analysis also for the ones who had remained microalbuminuric ( $n = 70$ ) ([Supplementary Table S2](#)). None of the 103 cases were used as controls.

In patients with microalbuminuria at study initiation visit (cases), we also evaluated whether allocation to intensive insulin therapy had an effect on uAOG/creatinine as compared to conventional insulin therapy.

At the DCCT study, intensive treatment group was defined as participants who took insulin 3 or more times per day by injection or an insulin pump, and self-monitored their blood glucose levels 4 or more times a

**Table 1.** Characteristics of cases and controls and uAOG at the study visit

Clinical Parameters	Controls	Cases	P Value
	N = 103	N = 103	
Study visit (Years from start)	2 (0–8)	2 (0–8)	0.7
Age (yr)	27 (14–42)	27 (13–41)	0.91
Gender			>0.99
Males (%)	48 (46.6)	48 (46.6)	
Females (%)	55 (53.4)	55 (53.4)	
Disease duration (mo)	107 (17–201)	111 (12–233)	0.47
Treatment			>0.99
Intensive (%)	60 (58)	60 (58)	
Standard (%)	43 (42)	43 (42)	
GFR (ml/min/1.73 m <sup>2</sup> )	122.6 (82–150)	126.1 (95–156)	0.045
HbA1c (%)	7.82 (5–12)	8.05 (5–14)	0.034
SBP (mm Hg)	112 (90–150)	116 (92–180)	0.005
DBP (mm Hg)	76 (52–90)	72 (54–90)	0.34
AER (mg/24hr)	8.64 (4–28.8)	33.12 (4–158)	<0.01
Log AER (mg/24h)	2.15 (1.5–3.4)	3.5 (1.5–5.1)	<0.0001
uAOG (ng/mg)	4 (0.1–65.3)	6.65 (1–1069)	<0.01
Log uAOG (ng/mg)	1.4 (–2.3 to 4.2)	1.9 (0.02–6.98)	<0.0001

AER, albumin excretion rate; AOG, urinary angiotensinogen; DBP, diastolic blood pressure; GFR, glomerular filtration rate; HbA1c, glycated hemoglobin; Log, logarithmic transformation; SBP, systolic blood pressure.  
Analysis by Wilcoxon rank sum test.

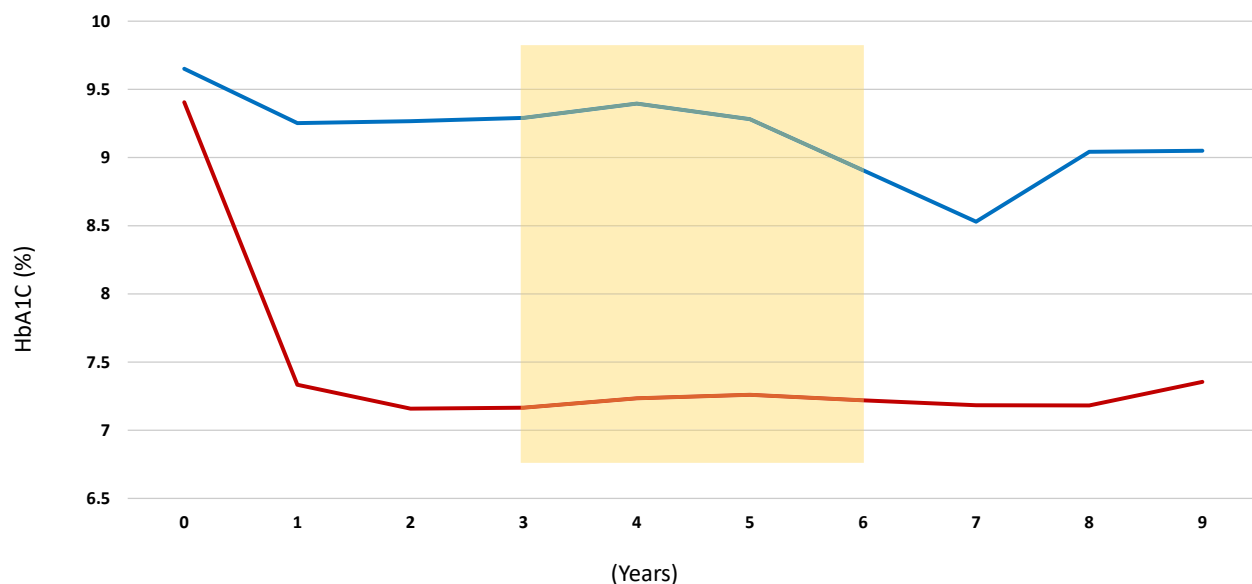
day, with ability to adjust dosage; and the conventional treatment group were participants using 1 or 2 injections of insulin a day, including mixed intermediate and rapid-acting insulins, with daily urine or blood glucose testing.<sup>40</sup>

Both groups had separated clearly in terms of hemoglobin A1c (HbA1c) during the first year of follow up (Figure 2). The visits chosen for these analyses were based on sample availability and duration of

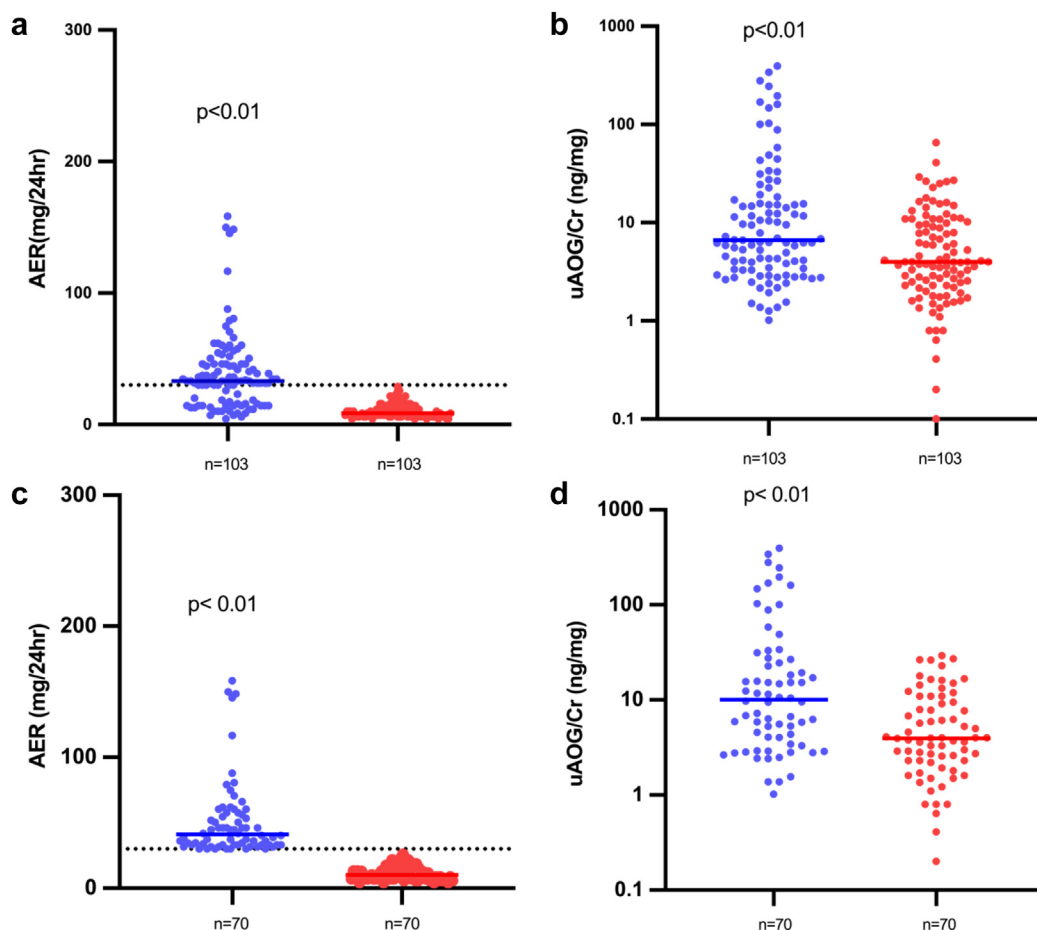
therapy of at least 3 years. Of the 103 subjects with microalbuminuria, only 99 had samples available for this analysis (from 41 subjects who received conventional and 58 subjects who received intensive insulin therapy and were matched based on age, gender, and disease duration).

### uAOG

The aliquots of urine were collected using standardized procedures and were frozen immediately and stored at  $-20^{\circ}\text{C}$  for up to only 2 weeks at the local clinic and then shipped frozen on dry ice to the Central Biochemistry Laboratory, where they remained frozen and thawed at  $-70^{\circ}\text{C}$  and later stored at the NIDDK repository at  $-80^{\circ}\text{C}$ . Available urine samples were transferred on dry ice to our laboratory at Northwestern University where it was immediately stored in  $-80^{\circ}\text{C}$  freezer. uAOG/creatinine was measured using a Human Total AOG Assay Kit from IBL (Japan). The AOG kit employed a solid-phase sandwich enzyme-linked immunosorbent assay whereby uAOG/creatinine was captured by one AOG antibody, which was coated onto the micro-titer plate. Horseradish peroxidase-conjugated AOG antibody was then added and tetramethylbenzidine was used as a chromogen. The reaction was stopped by the addition of sulfuric acid and the color intensity, which was proportional to the AOG concentration, was read using a 450 nm filter. The measurement range of the assay is 0.31 to 20 ng/ml (6.0–384.6 pmol/l). The interassay coefficient of variation for uAOG/creatinine was 5.7%, and the intra-assay



**Figure 1.** AER and urine AOG in 103 subjects in whom albumin excretion at the initial study DCCT visit was in the microalbuminuric range (upper panels) and in a subset of 70 of these subjects who continued to have microalbuminuria at the study visit (lower panels). (a) AER at the study visit in Cases (blue) and in normoalbuminuric Controls (red). (b and d) Show the corresponding uAOG values at the same study visit when the AER data (panels a and c) was available. AER, albumin excretion rate; uAOG, urine angiotensinogen.



**Figure 2.** HbA1c trend in subjects with microalbuminuria allocated to intensive (red) or conventional Insulin groups (blue). Highlighted is the follow up period used in this study to compare these 2 groups as HbA1c stabilized.

coefficient of variation was 4.4% ( $n = 100$  measurement). After the DCCT samples arrived at the Northwestern University laboratory, urine sample thawing and freezing was kept to the minimum (1–2 cycles if repeat measurements were required). uAOG measurements were minimally affected after 3 or 5 freeze-thaw cycles (96% and 92% of 1 freeze-thaw reference 20). The quality of DCCT samples is further suggested from the myriad of analytes measured in urinary and plasma samples.<sup>41–44</sup> Sample stability in terms of AOG in urine, however, was specifically addressed in a study that used urine from T1D patients stored for up to 3 years.<sup>20</sup> They found that intraindividual variation over 12-month for AOG was comparable to that of albumin, which is generally viewed as a stable urinary protein. After a 6-hour incubation at room temperature, a condition considered unfavorable for protein stability, AOG was very stable (89% of the reference) and, even after 48 hours, AOG was detectable at more than 60% of reference. Moreover, uAOG was minimally reduced (92% of reference) after 5 freeze–thaw cycles.

Data on 24-hour urinary albumin excretion and estimated glomerular filtration rate (GFR), calculated

using the chronic kidney disease-EPI formula, was provided to us by the DCCT repository at NIDDK.

### Statistical Analysis

For cases and controls with data at multiple time points, comparison was done using a signed rank test. The sample size of 104 matched cases and controls had a prespecified 80% power to detect a mean difference (between cases and controls) that was 0.27 standard deviations, assuming a 2-tailed test and a type I error rate of 5%.

To evaluate the normality of data distribution, Shapiro-Wilk test was used. For non-normally distributed data, Wilcoxon test was used to test the differences between unadjusted medians in the cases and controls. For data with normal distribution, *t*-tests were used. For testing the associations, multiple linear regression analysis was performed. Because the values of uAOG/creatinine and AER were not normally distributed, we transformed them into logarithmic values using natural logarithm.<sup>45</sup> A *P*-value < 0.05 was considered statistically significant. No corrections were made for multiple analyses.



## RESULTS

### uAOG in Patients With Microalbuminuria (Cases) and Normoalbuminuria (Controls)

Because of the regression to normoalbuminuria in 33 (32%) participants in the case group, the range of AER showed overlap among the cases (Figure 1a). The characteristics of the 103 cases at the study visit and those of the 103 controls matched for age, gender, disease duration at the study visit and allocation to treatment groups are shown in Table 1. There were small but significant differences in GFR, HbA1c, and systolic blood pressure (SBP) (Table 1). These differences were already present between both groups at the DCCT study initiation visit (Supplementary Table S1). By study design, AER was higher in cases than in controls (Table 1 and Figure 2).

The uAOG/creatinine ratio was significantly higher in samples from cases than in controls (median, 6.65 [1.0–1069] vs. 4.0 [0.1–65] ng/mg,  $P < 0.01$ ) (Figure 1a). After adjusting for SBP, diastolic blood pressure (DBP), HbA1c, and GFR, the logarithmically transformed uAOG/creatinine ratio remained significantly higher in cases than in controls ( $P = 0.01$ ). If AER is included in this analysis, however, the logarithmically transformed uAOG/creatinine ratio is no longer significant between the 2 groups ( $P = 0.14$  also by multiple linear regression analysis).

### uAOG in Cases in Whom AER Remained in the Microalbuminuric Range

Because at the study visit only 70 of 103 cases (68%) had AER in the microalbuminuric range and the remaining 33 had values in the normal range as noted above, we performed an additional analysis of the 70 cases and 70 matched controls who remained microalbuminuric (Supplementary Table S2). In this subset, AER levels in cases and controls were 41 mg/24h (range, 30.2–158 mg/24h) and 10 mg/24h (range, 4.3–23.0 mg/24h), respectively. Therefore, there was no overlap in AER between the cases and controls (Figure 1c).

The uAOG/creatinine ratio was higher in these 70 cases than in the 70 matched controls (10.03 vs. 3.95 ng/mg,  $P < 0.01$ ) (Supplementary Table S2 and Figure 1d). As in the full sample of 103 subjects per group, there were small but significant differences in GFR, HbA1c and SBP between cases and controls (Supplementary Table S2). After adjusting for SBP, DBP, HbA1c, GFR, the log uAOG/creatinine was significant ( $P = 0.0001$  by multiple linear regression analysis). If AER is included with GFR, SBP, DBP, HbA1c, age, sex, duration and treatment group, the log of uAOG/creatinine was still significantly higher ( $P = 0.046$  by multiple linear regression analysis).

In the remaining 33 cases with microalbuminuria at study initiation in whom AER had reverted to the normalalbuminuric range (14.4 [4.32–25.9] mg/24h), the uAOG/creatinine ratio was also markedly lower than in the 70 cases that had remained microalbuminuric (4.3 [1.26–44.4] vs. 10.03 [1.02–1067] ng/mg,  $P = 0.006$ ). When adjusted for SBP, DBP, HbA1c, and GFR, the logarithmically transformed uAOG/creatinine remained significantly higher by multiple linear regression analysis ( $P = 0.005$ ). If AER is included in this analysis, then the log uAOG/creatinine is no longer significant ( $P = 0.19$  by multiple linear regression analysis), which is consistent with the comparison of cases and controls in Table 1.

### uAOG in Males and Females

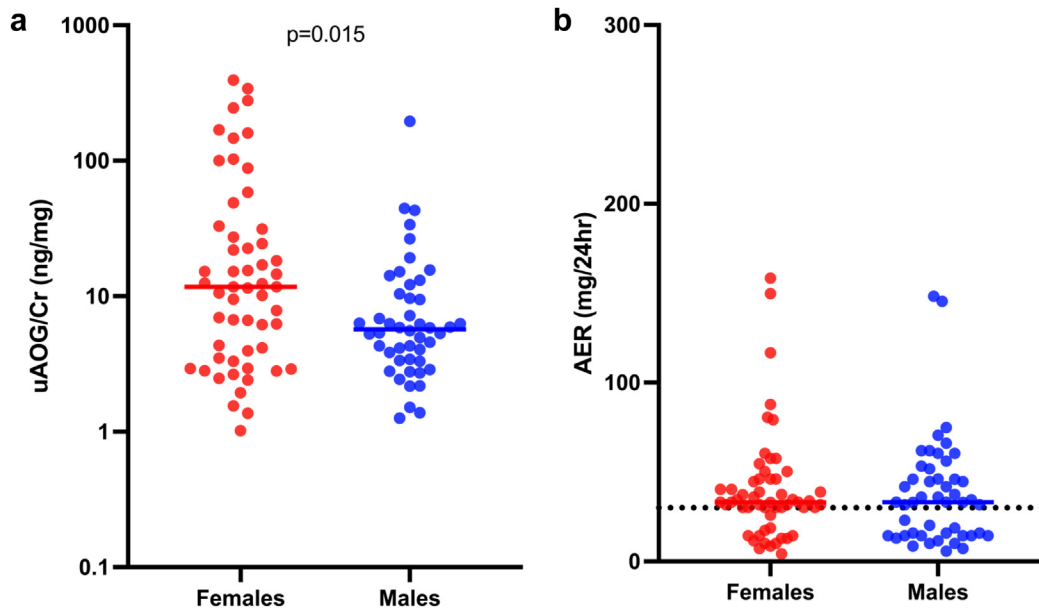
Among the cases ( $N = 103$ ), the uAOG/creatinine was markedly higher in females ( $n = 55$ ) than males ( $n = 48$ ) (11.73 [1.0–393] vs. 5.44 [1.0–1069] ng/mg,  $P = 0.015$ ) (Figure 3a). This difference in AOG was observed in the absence of significant differences in age, disease duration, allocation to treatment groups, GFR, HbA1c, and DBP but SBP was lower in females than in males (112 vs. 120 mmHg,  $P = 0.003$ ) (Supplementary Table S3). Of note, this difference was observed when AER was very similar in males and females (33.12 vs. 33.12 mg/24h,  $P = 0.9$ ) (Figure 3b).

After adjusting for all variables (AER, SBP, DBP, HbA1c, GFR, age, duration and insulin treatment allocation), the logarithmically transformed uAOG/creatinine was still significantly higher ( $P = 0.025$ , by multiple linear regression analysis).

In controls uAOG/creatinine was also higher in females than in males but the difference was not statistically significant (5.25 vs. 3.55 ng/mg,  $P = 0.12$ ). When comparing females in cases ( $n = 55$ ) with females in controls ( $n = 55$ ), uAOG/creatinine was significantly higher in cases than in controls (11.73 vs. 5.25 ng/mg,  $P = 0.002$ ). Males in cases ( $n = 48$ ) likewise had significant higher levels of uAOG/creatinine than males in controls ( $n = 48$ ) (5.44 vs. 3.55 ng/mg,  $P = 0.039$ ).

### uAOG in Patients Allocated to Conventional Versus Intensive Insulin Treatment

Of the 103 cases, only 99 had urine samples available for this analysis (see Methods). Fifty-eight samples were from patients allocated to intensive therapy and 41 from those allocated to conventional therapy (Table 2). The 2 groups had separated in terms of HbA1c clearly at the end of the first year of follow up but the visit chosen for uAOG measurements was based on sample availability during year 3 to year 6 (median, year 5) (Figure 2). There were no significant differences between the 2 groups in age, gender,



**Figure 3.** (a) Urinary AOG levels and (b) AER in patients with microalbuminuria (Cases) at the study entry (48 Males and 55 females). AER, albumin excretion rate; uAOG, urine angiotensinogen.

disease duration, GFR, and systolic or DBP (Table 2). As expected, participants in the intensive treatment group had markedly lower levels of HbA1c than patients in the conventional treatment (7.05 vs. 8.9%,  $P < 0.01$ ).

uAOG/creatinine was significantly lower in urine samples from cases in the intensive than in the conventional insulin treatment group (median, 3.98 [0.4–96] vs. 7.42 [1.4–296] ng/mg,  $P < 0.01$ ) (Figure 4a). After adjustment for GFR, SBP, DBP, AER, age, sex, and duration, the logarithmically transformed of uAOG/creatinine remained significantly lower by multiple linear regression analysis ( $P = 0.015$ ). Of note, AER excretion was not significantly different between conventional versus intensive insulin therapy (21.6 vs. 20.16 mg/24h,  $P = 0.68$ ) (Figure 4b).

As expected from the effect of the type of insulin therapy on HbA1c, when this parameter is included with GFR, SBP, DBP, AER, age, sex, and duration, the log uAOG/creatinine is no longer significant by multiple linear regression analysis ( $P = 0.66$ ). uAOG/creatinine was higher in females than in males in the conventional insulin arm (11.1 vs. 5.3 ng/mg,  $P = 0.04$ ) and in the intensive arm (5.3 vs. 2.4 ng/mg,  $P = 0.001$ ). These findings are shown in Supplementary Figure S2A and S2B. In the normoalbuminuric group (controls), uAOG/creatinine, in the intensive group was not significantly different from the conventional group (4 vs. 4.76 ng/mg,  $P = 0.77$ ) (Supplementary Table S4). Participants in the intensive treatment arm had markedly lower levels of HbA1c than patients in the conventional treatment arm (7.16 vs. 8.82%,  $P < 0.0001$ ).

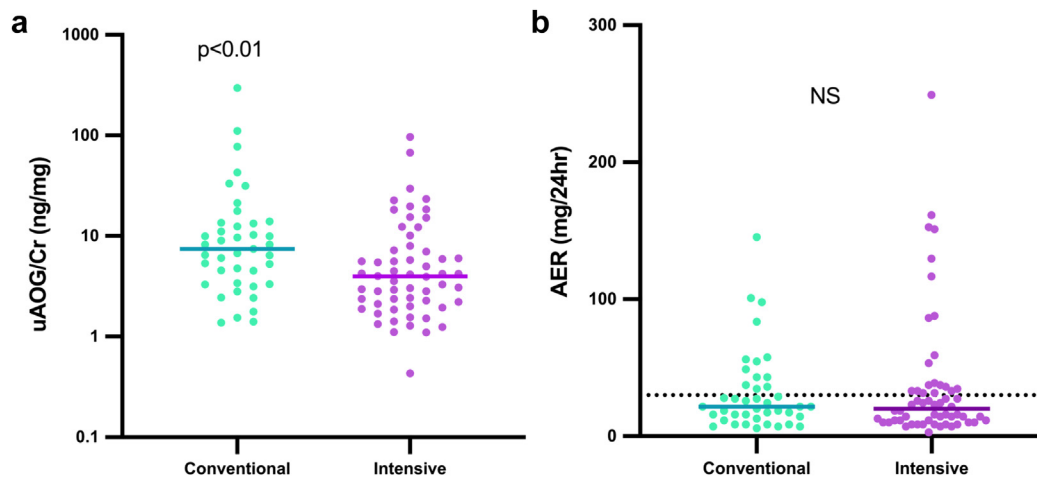
## DISCUSSION

This study examined uAOG in individuals with T1D who had microalbuminuria, the potential effect of gender

**Table 2.** Urine AOG in cases allocated to conventional or intensive insulin treatment for at least 3 years

Clinical Parameters	Conventional <i>n</i> = 41	Intensive <i>n</i> = 58	<i>P</i> Value
median (range)			
Visit	5	5	0.39
(Years from start)	(3–6)	(3–6)	
Age (yr)	30	30	0.94
	(18–43)	(18–44)	
Gender			0.92
Males (%)	18 (44)	26 (45)	
Females (%)	23 (56)	38 (55)	
Disease duration (mo)	129	164	0.19
	(63–245)	(65–252)	
GFR (ml/min/1.73 m <sup>2</sup> )	122.9	121.07	0.48
	(86.34–161)	(96.61–147)	
HbA1C (%)	8.9	7.05	<0.01
	(6.5–12.6)	(5.7–9.2)	
SBP (mm Hg)	116	117	0.96
	(90–140)	(90–158)	
DBP (mm Hg)	74	70	0.11
	(54–88)	(54–88)	
AER (mg/24h)	21.6	20.16	0.68
	(5.8–145)	(2.9–249)	
Log AER (mg/24h)	3.07	3	0.68
	(1.8–4.98)	(1.1–5.5)	
uAOG (ng/mg)	7.42	3.98	<0.01
	(1.4–296)	(0.4–96)	
Log uAOG (ng/mg)	2	1.38	0.0047
	(0.31–5.69)	(–0.84 to 4.57)	

AER, albumin excretion rate; AOG, urinary angiotensinogen; DBP, diastolic blood pressure; GFR, glomerular filtration rate; HbA1c, glycated hemoglobin; Log, logarithmic transformation; SBP, systolic blood pressure. Analysis by Wilcoxon rank sum test.



**Figure 4.** (a) Urine AOG and (b) AER in the conventional versus intensive insulin treatment group at the same study visit more than 3 years poststudy initiation (see Figure 1). AER, albumin excretion rate; uAOG, urine angiotensinogen.

differences and the potential effect of intensive insulin therapy as compared to conventional insulin therapy. We used urine samples from the NIDDK repository from participants in the DCCT who had microalbuminuria at the study initiation and controls who were normoalbuminuric throughout the study. The first finding was that in participants who had AER in the microalbuminuric range at the study initiation, uAOG (measured at the earliest study visit when urine was available, on average 2 years post study initiation) was increased as compared to matched controls in whom AER was in the normal range. On further analysis this difference in uAOG was not seen when AER had regressed to the normal range in 33 of the 103 cases who initially presented with microalbuminuria. Consistent with our findings, the rate of conversion of microalbuminuria to normoalbuminuria in T1D has previously been reported to occur in about one-third of patients with T1D.<sup>46,47</sup> A similar conversion in uAOG can be inferred from our study but cannot be established because sequential urine samples were not available.

The finding that AOG is increased in patients with T1D with microalbuminuria, though not new, is relevant because of the unique characteristics of our cohort. Specifically, the increase in uAOG was noted very early in the course of T1D and in the absence of medications that could alter AOG, such as RAS blockers. AOG has been reported to be increased in the urine of patients with type 1 and type 2 diabetes who had albuminuria in the microalbuminuric or macroalbuminuric range.<sup>19–21,27,48</sup> These previous studies, however, included patients with hypertension and they were treated with antihypertensives, including RAS blockers. In our study, all the patients with T1D were not treated with RAS blockers for renoprotection, because these agents were not used during the DCCT.

This is important, because uAOG can be decreased by RAS blockers and can be increased in patients with hypertension.<sup>27,49–51</sup> Study participants were not overtly hypertensive, but it should be noted that even early in the course of T1D, blood pressure may be slightly elevated, particularly at night time as assessed by 24 hour ambulatory blood pressure.<sup>52</sup> In our study, office SBP was slightly higher in patients with microalbuminuria than in those with normoalbuminuria. After adjusting for SBP and DBP, uAOG still remained higher in subjects in the microalbuminuric group (103 cases) than in normoalbuminuric group (103 controls).

Altogether, the data show that in the microalbuminuric stage of T1D, independently of blood pressure and use of RAS blockers, uAOG excretion, estimated by the uAOG/creatinine ratio, is increased. Furthermore, the uAOG/creatinine was much lower in the 33 subjects in whom AER had returned to normal as compared to the 70 in whom microalbuminuria had persisted. This suggests that the glomerular handling of uAOG by the kidney is similar to that of albumin, consistent with the fact that both proteins, are similar in molecular size and therefore affected by the glomerular injury that occurs early in T1D. Our study moreover unraveled that gender and type of insulin therapy can influence uAOG independently of AER and glomerular injury as discussed in the next paragraph.

That among subjects with T1D and microalbuminuria, uAOG/creatinine is higher in females than males, to our knowledge, has not been previously described. AER was not different between males and females, suggesting different production rates of AOG rather than enhanced glomerular passage of AOG. The higher levels of uAOG in females than in males with

microalbuminuria, particularly in a young group of females such as the participants in this study, is most likely attributable to estrogen driven AOG synthesis. It is known that estrogens increase uAOG/creatinine.<sup>53</sup> Estrogens increase transcription of the AOG gene likely from a direct interaction between the 5'-flanking region of the gene and the DNA-binding domains of their cognate receptors.<sup>54</sup> Though we did not have sufficient plasma samples to measure AOG, it has been reported that in rat plasma, AOG is higher in females than in males, consistent with increased liver synthesis of AOG.<sup>55</sup> In addition, estrogens may induce intrarenal production of AOG.<sup>56</sup> Therefore, we surmise that higher levels of uAOG found in females with T1D largely reflects increased production of AOG by the liver, the kidney, or both.

uAOG was lower in cases allocated to intensive insulin therapy than in those in the conventional insulin therapy, a finding that is novel in humans and that unravels a possible effect of insulin on uAOG and increased tubular synthesis because increased glomerular passage alone would be expected to affect AER and AOG to roughly the same extent.

The source of uAOG has been debated.<sup>14–16,18,28,57,58</sup> Though circulating liver-derived AOG is likely the main source,<sup>18</sup> AOG produced in the proximal tubules of the kidney contributes to uAOG as well.<sup>14–16,28,57</sup> In the absence of kidney disease, the glomerular passage of AOG and albumin occurs, albeit in small amounts,<sup>16</sup> and then both proteins are reabsorbed in the proximal tubule,<sup>59,60</sup> so normally the amount found in the urine is very low. Therefore, any increases in uAOG in the setting of increased albumin excretion could reflect alterations in the glomerular filtration barrier, impaired proximal tubular reabsorption, or both. This, however, does not exclude the possibility that increased uAOG also reflects increased production of AOG by the liver or the kidney. In fact, the higher levels of AOG in females than in males with microalbuminuria, and in subjects on conventional than those on intensive insulin therapy suggests that AOG production by the liver, the kidney or both can contribute to uAOG in T1D. This becomes apparent in the microalbuminuric phase of the disease, and it is not seen while AOG and AER are excreted in smaller amounts as in normoalbuminuric subjects.

We surmise that the finding of reduced uAOG/creatinine by intensive insulin therapy as compared to conventional insulin therapy is best explained by changes in AOG production by the kidney because there were not significant changes in albumin excretion between the 2 groups (Table 2). This postulation is based on experimental work showing that formation of AOG in the proximal tubule is regulated by insulin via 2 transcriptional factors (hnRNP F and hnRNP K) that

normally repress kidney AOG synthesis.<sup>30</sup> Stimulation of these ribonucleoproteins by sustained levels of insulin in the intensive group may therefore explain the lower levels of uAOG as a result of decreased AOG synthesis. The improved metabolic control associated with intensive insulin additionally may suppress AOG synthesis because it is well known that high glucose also increases AOG synthesis in cell models.<sup>61,62</sup> Downregulation of AOG synthesis should lead to decreased formation of Angiotensin II, the main biologic peptide, in the proximal tubule.<sup>63,64</sup> It is therefore possible that the well-known renoprotective effect of improved glycemic control using intensive insulin regimens is due, at least in part, to downregulation of kidney AOG and consequently less activation of the local kidney RAS.

It can also be inferred that a high level of uAOG could be a biomarker of DKD progression. In a previous longitudinal study, we showed that in people with T1D uAOG increases before the development of stage 3 chronic kidney disease.<sup>65</sup> Increased uAOG was also associated with progressive renal decline, an index of progression of end stage kidney disease.<sup>65</sup> The increased uAOG/creatinine may constitute a complementary biomarker of DKD progression, which provides information on the status of the RAS within the kidney. A finding of increased levels of uAOG/creatinine may therefore provide a rationale for more intensive insulin therapy and/or initiation of RAS blockers early in the course of the disease.

In summary, uAOG/creatinine is increased in patients with T1D and microalbuminuria and the level is higher in females than in males, likely reflecting increased AOG production. Intensive insulin therapy and its associated improved glycemic control are associated with reduced uAOG, possibly reflecting decreased intrarenal AOG synthesis as a contributing mechanism to renoprotection.

## DISCLOSURE

DB and JW are coinventors of patents entitled "Active low molecular weight variants of angiotensin converting enzyme 2 (ACE2)," "Active low molecular weight variants of angiotensin converting enzyme 2 (ACE2) for the treatment of diseases and conditions of the eye," and "Soluble ACE2 variants and uses therefor." DB is founder of Angiotensin Therapeutics, Inc. DB received consulting fees from AstraZeneca and Advicenne Inc., all unrelated to this work. During the conduct of these studies, DB received unrelated support from a grant from AstraZeneca and research funding from the Feinberg Foundation. All other authors declare no conflicting interests.



## ACKNOWLEDGMENTS

DB is the guarantor of this paper taking full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

## Funding

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant R01DK104785 and by National Institutes of Health National Institute of Allergy and Infectious Diseases [Grant 1R21AI166940-01] as well as by a gift to Northwestern University by the Joseph and Bessie Feinberg Foundation (DB).

## SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

**Figure S1.** Albumin excretion rate (AER) at the DCCT study initiation visit in 103 patients with microalbuminuria (cases) and 103 patients with normoalbuminuria (Controls).

**Figure S2.** Urine AOG levels in males and females with microalbuminuria in the intensive and conventional insulin therapy group.

**Table S1.** Characteristics of cases and controls at study initiation visit.

**Table S2.** Subset analysis restricted to cases with persistent microalbuminuria at study visit and controls.

**Table S3.** Characteristics males and females cases with microalbuminuria.

**Table S4.** Characteristics of controls in the conventional versus intensive treatment in normoalbuminuric patients.

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