The Relationship between Urinary Renin Angiotensin System Markers, Renal Function and Blood Pressure in Adolescents with Type 1 Diabetes

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Running Title: RAAS markers in adolescents with type 1 diabetes

Word Count: 3,546

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ABSTRACT:

Aims: The relationship between the renal renin-angiotensin aldosterone system (RAAS) and cardiorenal pathophysiology is unclear. Our aims were to assess (1) levels of urinary RAAS components and (2) the association between RAAS components and HbA1c, urine albumin/creatinine ratio (ACR), estimated glomerular filtration rate (eGFR) and blood pressure in otherwise healthy adolescents with type 1 diabetes mellitus (TID) vs. healthy controls (HC).

Methods: Urinary angiotensinogen and ACE2 levels, activity of ACE and ACE2, blood pressure (BP), HbA1c, ACR and eGFR were measured in 65 HC and 194 T1D from the Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial (AdDIT).

Results: Urinary levels of all RAAS components were higher in T1D vs. HC ($p<0.0001$). Higher HbA1c was associated with higher urinary angiotensinogen, ACE2, and higher activity of ACE and ACE2 ($p<0.0001$, $p=0.0003$, $p=0.003$ and $p=0.007$ respectively) in T1D. Higher ACR (within the normal range) was associated with higher urinary angiotensinogen ($p<0.0001$) and ACE activity ($p=0.007$), but not with urinary ACE2 activity or ACE2 levels. These observations were absent in HC. Urinary RAAS components were not associated with BP or eGFR in T1D or HC.

Conclusions: Otherwise healthy adolescents with T1D exhibit higher levels of urinary RAAS components compared to HC. While levels of all urinary RAAS components correlate with HbA1c in T1D, only urinary angiotensinogen and ACE activity correlate with ACR, suggesting that these factors reflect an intermediary pathogenic link between hyperglycemia and albuminuria within the normal range.

Key Words: type 1 diabetes, ACE, ACE2, hyperglycemia, albumin to creatinine ratio

ABBREVIATIONS:

ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
ACR	Albumin to Creatinine Ratio
AdDIT	Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial
Ang I	Angiotensin I
Ang II	Angiotensin II
BMI	Body Mass Index
BP	Blood Pressure
CV	Coefficient of Variation
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
eGFR	Estimated Glomerular Filtration Rate
HbA1c	Hemoglobin A1c
HC	Healthy Controls
HDL cholesterol	High-Density Lipoprotein Cholesterol
HR	Heart Rate
LDL cholesterol	Low-Density Lipoprotein Cholesterol
MAP	Mean Arterial Pressure
NS	Not Statistically Significant
RAAS	Renin-Angiotensin Aldosterone System
SBP	Systolic Blood Pressure
SD	Standard Deviation

TID	Type 1 Diabetes Mellitus
TID-H	Type 1 Diabetes Mellitus - Hyperfiltration
TID-N	Type 1 Diabetes Mellitus - Normofiltration
T2D	Type 2 Diabetes Mellitus

INTRODUCTION:

While systemic components of the renin-angiotensin aldosterone system (RAAS) are downregulated in diabetes mellitus (DM) (21), the intrarenal RAAS is activated thereby playing an important role in the pathogenesis of diabetic nephropathy through increased intraglomerular pressure and hyperfiltration, and stimulation of tubulointerstitial fibrosis (4). Increased angiotensinogen produced in the proximal tubule cells is converted to Angiotensin I (Ang I) by renin (11). Subsequently, the abundance of angiotensin converting enzyme (ACE) in the proximal tubule cells favours the conversion of Ang I to Ang II. Increased intrarenal Ang II is associated with diabetic nephropathy in rats (12), which chronically induces hyperfiltration, proteinuria and injury to glomerular endothelium, basement membrane and podocytes (36). Angiotensin-converting enzyme 2 (ACE2) is another component of the RAAS that degrades angiotensin II (Ang II) to the Ang(1-7) fragment (28). Similar to other RAAS components, ACE2 is highly expressed in the kidney (28). However, in contrast with Ang II, ACE2 may be renal protective (41). As reviewed elsewhere, deletion of the *Ace2* gene leads to the activation of oxidative stress pathways (38) and the development of *de novo* glomerulosclerosis in mice (27) and pharmacologic inhibition of ACE2 worsens experimental diabetic nephropathy (29).

Given the role of RAAS activation in the pathogenesis of diabetic nephropathy, the quantification of intrarenal RAAS activation in humans may help to identify patients at higher risk of renal complications. RAAS components, such as angiotensinogen and ACE2, are detectable in human urine (19) and serum (33) and urinary enzymatic activity of ACE and ACE2 can also be measured (6). Urinary angiotensinogen is increased in patients with type 2 diabetes (T2D) compared to healthy controls (HC), and progressively increases as patients transition from normo- to micro- to macro-albuminuria (25). Similarly, in small studies involving children and

young adults with type 1 diabetes (T1D) urinary angiotensinogen levels are increased compared to HC (23, 30). Moreover, angiotensinogen levels in children with T1D are positively associated with ambulatory blood pressure (30). Urine ACE2 protein and activity levels have also been shown to be elevated in adults with uncomplicated T1D (6). In the setting of chronic kidney disease, renal transplant patients with diabetes and patients with heart failure exhibit higher urinary ACE2 protein levels (14, 33, 39). In adults with T2D, Park et al have further demonstrated that urinary ACE2 is increased and independently associated with microalbuminuria (19). In contrast with what is known in adults, less is known about levels of urinary RAAS markers or their relationships with glycemic control or renal/cardiovascular function in cohorts with even earlier subclinical disease, such as in adolescents with T1D.

The objective of this study was to determine if urinary excretion of angiotensinogen and ACE2 protein, as well as urinary ACE and ACE2 enzymatic activity, is elevated in adolescents with uncomplicated T1D compared to HC. We hypothesized that urinary excretion of RAAS markers would be elevated in adolescents with T1D compared to HC and that levels would be further elevated in T1D patients with renal hyperfiltration (eGFR≥135 ml/min/1.73m^2). We also hypothesized that the highest tertile of ACR within the normal range would be associated with the highest levels of these RAAS markers.

RESEARCH DESIGN AND METHODS:

Study Population

This was a cross-sectional study involving patients who were recruited from the longitudinal, observational, non-interventional arm of the AdDIT, from clinical sites in the Greater Toronto Area. The Non-Randomized Low-Risk arm of AdDIT is a 4-year

observational/natural history study, following adolescents at low and medium risk of developing microalbuminuria (EudraCT Number: 2007-001039-72). High-risk adolescents were recruited into the AdDIT Interventional Study (http://www.clinicaltrials.gov/ct2/show/NCT01581476), which was designed to examine the effect of ACE inhibitors and statins on clinical endpoints. Our cross-sectional study did not include participants involved in the intervention trial. However as an ancillary component of Non-Randomized Low-Risk arm of AdDIT we also included high-risk subjects who chose not to enter the AdDIT Intervention Study.

A cross-sectional analysis was conducted using the blood and urine samples collected from 194 T1D and 65 HC participants from the AdDIT trial (1). Inclusion/exclusion criteria have been described elsewhere (8). In brief, the study population consists of 11 to 16 years old adolescents inclusively, who achieved a minimum of Tanner stage 2 for puberty and could not be taking anti-hypertensive or lipid-lowering agents or medications that interfere with the RAAS. All patients were on a multiple insulin dose regimen or on an insulin pump at the time of the screening visit. HC were recruited through local advertisements as similar aged volunteers, who were not on any vasoactive medications, had no previous history of familial hyperlipidemia, diabetes, obesity, hypertension, or any other significant cardiac, renal or systemic disease and normal cardiac anatomy and function by screening echocardiogram, as described elsewhere (8). The Hospital for Sick Children Research Ethics Board, Credit Valley Hospital Ethics Forum and Markham-Stouffville Research Ethics Board approved the protocol and the consent procedure. Written informed consent was obtained from the legal guardian/next of kin/caretakers of minors aged 15 and younger, while the minors provided assent. All subjects aged 16 with capacity to understand the study information, gave complete written and informed consent to participate in the study.

Clinical Assessment

For adolescents with T1D, data on chronological age, age at diabetes onset, and duration of diabetes were collected. For all subjects, height was measured by a wall-mounted stadiometer and weight by electronic scales and resting heart rate and right brachial blood pressure was measured using an age-appropriate cuff and averaging 3 readings with an automated DINAMAP® sphygmomanometer (Critikon, Tampa, Florida, USA). Systolic and diastolic blood pressures (SBP and DBP respectively) were also converted to z-scores for age, sex and height. For all 194 T1D and 65 HC that underwent the same baseline clinical assessment, fasting blood samples were collected for glucose, HbA1c measurements, lipid profiles (total cholesterol, HDL [high-density lipoprotein] cholesterol, LDL [low-density lipoprotein] cholesterol and triglycerides) and serum cystatin C was used to calculate estimated glomerular filtration rate (eGFR) (8).

Sample Collection and Analytical Methods

The enzyme activities of urinary ACE2 and ACE were measured using synthetic substrates as previously reported (39). The amount of ACE2 present in urine specimens was quantified using a commercial ELISA kit (Cat. No. AG-45A- 0022EK-KI01, AdipoGen, Seoul, Korea) according to the protocol provided by the supplier (http://www.adipogen.com/ag-45a-0022/ace2-human-elisa-kit.html). A standard curve was generated by performing 1:2 serial dilutions of human recombinant ACE2 (50 ng/ml), provided with the kit, with the limit of detection ranging from 0.391 to 25 ng/mL. In preliminary experiments, the average intra-assay coefficient of variation (CV) for the assay was 2.9%, and the average inter-assay CV was 8.7%. For urinary angiotensinogen measurements, a commercial ELISA kit was used (Immuno-

Biological Laboratories Co., Ltd., Takasaki-Shi, Gunma, Japan; Code No. 27412), with a detection limit from 0.313 ng/mL to ~20 ng/mL. In preliminary experiments, the intra-assay CV was 3.46%, and inter-assay CV was 7.93%. All urinary RAAS measures were corrected for urine creatinine concentration.

Renal Assessments

The mean of 2 ACR measures obtained from 2 sets of 3 early-morning urine samples was obtained and adjusted on a log ACR scale using age, diabetes duration, sex and the coefficients from the ORPS linear regression model (1). The T1D participants were divided into the following adjusted ACR tertiles: (1) 64 patients in low ACR tertile (<0.8 mg/mmol), (2) 77 patients in the middle ACR tertile (0.8-1.2 mg/mmol), and (3) 53 patients in the high ACR tertile (>1.2 mg/mmol). The tertile boundaries were determined based on preliminary data from the ORPS cohort which predicted the risk for development of microalbuminuria (1). All urine and blood samples were obtained during the screening phase of the study. As in our previous work, eGFR was calculated using the Larsson's formula GFR = 77.24 x Cys $C^{-1.2623}$, where cystatin C was measured by laser immunonephelometry (Dade Behring) (8). T1D adolescent participants were also subdivided into a normofiltration (TID-N) and a hyperfiltration (TID-H) group, where hyperfiltration was defined as eGFR ≥135 mL/min/1.73m^2.

Statistical Analysis

Normally distributed data are presented as mean ± standard deviation (SD). Non-normally distributed data are presented as median and interquartile range. RAAS markers were log transformed in order to stabilize the variance. *Between-group* comparisons of baseline

parameters in T1D vs. HC groups were made using t-tests for normally distributed data and the Mann-Whitney U test was used for non-normally distributed data. To determine the association between the RAAS parameters with blood glucose, HbA1c and ACR, a multivariate regression analysis was first used. About 30% of patients in our cohort had undetectable RAAS marker measurements. Replacing a large number of undetectable values with zero could lead to a downward biased estimate of the slope coefficient in the regression analysis. We therefore used a TOBIT analysis, which is a censored regression model designed to estimate linear relationship between variables taking into account the undetectable dependent variable (7). The TOBIT analysis adjusted for below detection levels of respective RAAS markers along with adjustment for age, gender, BMI z score, T1D duration and HDL cholesterol (7). Spearman correlation coefficient was used to determine an association between non-normal outcomes. Statistical significance was defined as $p<0.0125$ to account for the effect of multiple comparisons. All statistical analyses were performed using SAS v9.4 and GraphPad Prism software (version 5.0).

RESULTS:

Baseline characteristics

The 194 T1D and 65 HC adolescent participants included in this cross-sectional analysis from the Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial (AdDIT) were normotensive and normoalbuminuric. Baseline parameters, such as gender distribution, age, blood pressure, eGFR and ACR were similar between HC and T1D adolescents (Table 1). T1D participants had a higher z-score body mass index compared to HC. Of the 194 T1D patients, 132 exhibited normofiltration (68%) and 62 hyperfiltration (32%). HbA1c, plasma glucose and plasma HDL cholesterol were higher in T1D compared to HC.

Urinary levels of ACE2 and angiotensinogen and enzyme activity of ACE and ACE2 in the HC and T1D cohorts

Urinary ACE2 activity and urinary ACE2 protein levels, as well as ACE activity and angiotensinogen were elevated in the T1D group vs. HC (Table 1, Figure 1). There were no significant differences observed in the RAAS components between the 3 ACR tertiles (Figure 2) or when comparing T1D-N and T1D-H groups (Figure 3).

Urinary RAAS markers, plasma glucose and HbA1c in HC and T1D cohorts

In the T1D cohort, higher plasma glucose at the time of urine sample collection correlated with log urinary ACE activity (β=0.03, $p<0.0001$) (Table 2). Higher HbA1c was also correlated with higher log urinary angiotensinogen (β=0.14, $p<0.0001$), log urinary ACE activity (β=0.80, $p=0.003$), log urinary ACE2 activity (β=0.31, $p=0.007$) and urinary ACE2 levels (β=0.13, $p=0.0003$). These associations were significant after correcting for below detection levels of RAAS markers, age, gender, BMI z-score, T1D duration and HDL cholesterol. None of the relationships were present in HC.

The relationship between urinary RAAS components with ACR, renal function and blood pressure in the T1D and HC cohorts

After correcting for below detection levels of RAAS markers, age, gender, BMI z-score, T1D duration and HDL cholesterol, higher log ACR levels were correlated with higher log angiotensinogen (β=0.50, $p<0.0001$, Table 2) and ACE activity (β=0.26, $p=0.007$) in T1D adolescents. In contrast, log ACE2 activity and log ACE2 protein levels were not significantly

associated with log ACR after correcting for the covariates mentioned above. None of these relationships were significant in the HC group. Urinary RAAS components were not associated with eGFR or blood pressure in either group (Table 3).

DISCUSSION:

Current methods to identify young patients with T1D at the highest risk of developing renal and cardiovascular complications remain limited prior to the onset of GFR decline, microalbuminuria or hypertension. It is perhaps for this reason that untargeted approaches using available agents such as ACE inhibitors and angiotensin receptor blockers in patients with uncomplicated disease have been unsuccessful, since the overall risk of complications over 5-10 years remains relatively low. It is therefore important to develop earlier pre-clinical markers of complications, so that higher risk patients can potentially be identified sooner after diagnosis and thereby targeted for earlier and perhaps even preventative therapies. It has been reported that the highest tertile of normoalbuminuria below the threshold for microalbuminuria in adolescents with T1D may identify a subgroup of patients at higher risk of subsequent progression to microalbuminria (13). However, the physiological factors that promote these early pre-clinical changes in urinary albumin excretion remain unclear.

Our first major novel observation in this large cohort of adolescent patients with T1D was the significantly higher levels of urinary ACE activity, ACE2 activity and ACE2 protein excretion in the T1D cohort compared to HC. In addition, as reported by Soltysiak et al in smaller cohort, urinary angiotensinogen levels were significantly higher in adolescents with T1D compared to HC (30). Previous studies have primarily focused on older patients with T2D, especially those with established nephropathy, and have reported that these patients have elevated levels of urinary RAAS markers. Previous reports have also demonstrated elevated

urinary RAAS mediator excretion rates in adults with T1D compared to HC (6). Although the clinical implications of this observation need to be further elucidated, higher levels of urinary RAAS markers may reflect intrarenal RAAS activation, potentially leading to effects on albumin excretion as described below. The observation that the urinary excretion of RAAS mediators was higher in T1D participants is especially important in light of the fact that serum levels of RAAS are consistently suppressed in patients with diabetes in the context intrarenal RAAS activation – a phenomenon called "the RAAS paradox" (3, 4, 14). While intrarenal activation of the RAAS despite low systemic levels is incompletely understood, hyperglycemia increases intrarenal angiotensinogen expression and angiotensin II levels through heterogeneous nuclear ribonucleoproteins F and K, reactive oxygen species and hexosamine pathway activation in renal tubular cells (10, 26, 35). Although we were not able to measure RAAS levels in blood in this analysis because of small volumes of blood samples taken in children, our results further reinforce the concept of distinct, directionally opposite levels of RAAS activation in the systemic circulation compared to the intrarenal compartment – suggesting that urinary levels do not simply reflect overflow from blood. Nevertheless, since angiotensinogen has a similar molecular weight as albumin and is also negatively charged, the positive correlation between urinary levels of angiotensinogen and albumin in adolescents with T1D may be related to similarities in glomerular handling, leading to increased excretion of both proteins (22).

Our second observation was that higher HbA1c at the time of the urine sample collection correlated with higher urinary angiotensinogen, ACE activity, ACE2 protein levels and ACE2 activity. Previous *in vitro* studies have demonstrated a relationship between hyperglycemia and activation of the RAAS through increased angiotensinogen and renin mRNA expression (11). In humans, Park et al previously demonstrated that urinary ACE2 levels are associated with

metabolic parameters such as HOMA-IR and fasting blood glucose (19). Urinary angiotensinogen and ACE mRNA expression in human renal biopsy tissue are similarly associated with poor glycemic control in patients with T2D (15, 17). Previous experimental work has suggested that the glucose-mediated stimulatory effect on angiotensinogen gene expression in renal proximal tubular cells is mediated by alterations in MAPK signalling, reactive oxygen species and hexosamine biosynthetic pathways (9). Finally, since pharmacologically-induced increased glycosuria during clamped *euglycemia* is associated with higher urinary excretion of RAAS markers, it is possible that it is the increase in glycosuria rather than hyperglycemia that increased the urinary excretion of RAAS markers (5). While we could not determine if increased urinary excretion of RAAS markers was due to the effect of ambient hyperglycemia or the consequent glycosuria (5), the positive correlation between HbA1c and urinary RAAS markers suggests a stimulatory effect on the intrarenal RAAS, which may increase albuminuria. Alternatively, it is possible that injury pathways induced by the diabetic milieu resulted in increased urinary loss of tubular cells, which express RAAS mediators. Future work should therefore determine whether or not urinary RAAS mediators reflect ongoing diabetes-related tubular cell injury (11).

The possible mechanisms leading to increased ACE2 excretion in urine requires additional comment. Previous work has demonstrated that shedding of ACE2 into the urine is mediated by ADAM-17, and is stimulated by Ang II and high glucose. This has been shown in cell culture (40), and the important role of glucose in ACE2 shedding has also been emphasized in studies in Akita mice *in vivo* (24). Therefore the correlation between HbA1C with urinary ACE2 levels in our dataset fits well with this model linking hyperglycemia with urinary ACE2. In this regard, the source of urinary ACE2 likely arises from tubular epithelial cells rather than

systemic filtration, as suggested by experimental studies (37). Future work should consider simultaneous measurements of blood and urine ACE2 levels to better define the source of urinary ACE2 excretion.

Our third major observation was that urinary angiotensinogen and ACE activity were associated with higher levels of albuminuria within the normal range in adolescents with T1D, while such an association was not observed with urinary ACE2 levels or activity. In animal models, deletion of the ACE2 gene exacerbates albuminuria, mesangial matrix deposition, glomerular basement membrane thickening and glomerulosclerosis and administration of human recombinant ACE2 or ANG-(1-7) reduces albuminuria, blood pressure, renal fibrosis, oxidative stress and levels of tissue inflammation (27, 29). In humans, previous studies have reported a significant increase in serum ACE2 activity in male patients with T1D with micro- or macroalbuminuria compared to HC or normoalbuminuric T1D patients (31). In patients with T2D and nephropathy, urinary and renal biopsy mRNA expression of ACE and urinary ACE2 levels are also associated with albuminuria (15, 19, 34), and urinary ACE2 levels are elevated in patients with diabetic nephropathy compared to patients without this complication (14). To our knowledge, the current report represents the first time that urinary angiotensinogen and ACE activity have been associated with ACR within the normal range in adolescents with T1D (42). Consistent with previous studies in other patient cohorts with varying severity of complications, urinary ACE2 levels and activity were not associated with albuminuria in T1D adolescent patients without complications (14, 31). Whether the positive correlation between urinary ACE2 and ACR in other patient cohorts with nephropathy, micro- or macroalbuminuria represents a deleterious effect of ACE2 shedding or instead a compensatory upregulation of this arm of the RAAS activation cascade in response to ACE-Ang II activation is not known. We hypothesize

that higher urinary ACE2 levels reflect compensatory activation of this pathway in patients with complications related to their diabetes, while such pathways are not yet activated in otherwise healthy patients with diabetes. In future work, it will therefore be important to determine the relationship between urinary RAAS markers and indices of renal risk over time, including the markers of tubular injury, the development of microalbuminuria and GFR slope.

In patients with T2D and baseline renal function impairment, urinary angiotensinogen and ACE mRNA expression obtained from renal biopsy samples are associated with lower eGFR; such an association is not seen in patients with preserved renal function (15, 17). Urinary ACE and ACE2 levels in patients with T2D also correlate with renal function impairment, and urinary ACE2 is associated with progressive eGFR decline (34). In contrast to previous observations in cohorts with renal impairment, in our otherwise healthy cohort of adolescents with T1D, we did not detect a correlation between eGFR and urinary RAAS markers. Furthermore, and in contrast with experimental models of diabetes (2, 3, 16, 32), patients with the earliest renal hemodynamic abnormality – renal hyperfiltration – did not exhibit higher levels of urinary RAAS markers. Therefore, the relationship between urinary RAAS markers and eGFR may be modified with a longer diabetes duration.

In light of the relationship between the RAAS and systemic vascular function in patients with T2D (17), (15), (19), we anticipated that higher levels of RAAS mediators would correlate with higher blood pressure. In our cohort of normotensive, normoalbuminuric adolescents with T1D, we did not observe a relationship between any of the RAAS markers and blood pressure or systemic vascular function. It is therefore tempting to speculate that the relationship between urinary RAAS markers and blood pressure may be modified over time with a longer duration of disease – a hypothesis that will be tested during the longitudinal follow up of this cohort.

Our work has limitations. First, we were only able to measure urinary and not plasma RAAS mediators, due to the limited volumes obtained in children. Second, our observations were made in adolescents. As such, we cannot comment about the generalizability of our findings to adults with preclinical disease, or to adolescent with other medical conditions such as T2D or other non-diabetic nephropathies. Additionally, although cystatin C based eGFR measurements may better identify acute changes in kidney function compared to creatinine-based methods (18), cystatin C still tends to underestimate GFR in the higher range compared to the gold standard inulin clearance based GFR measurement technique (20). Finally, this was a cross-sectional study with the analysis performed on samples taken on one occasion. Thus, we cannot predict changes that occur over time in individual patients and further studies are required to investigate clinical implications of our observations.

In conclusion, urinary levels of RAAS mediators are associated with glycemic burden and urinary angiotensinogen and ACE activity correlate with higher urine ACR. Further work is required to determine whether urinary RAAS markers can help to identify patients at higher risk of future renal and cardiovascular complications.

ACKNOWLEDGEMENTS:

D.Z.I.C. was also supported by a Kidney Foundation of Canada Scholarship and a Canadian Diabetes Association-KRESCENT Program Joint New Investigator Award and with funding from CIHR and the Kidney Foundation of Canada. K.D.B was supported by a research salary award from the Dept. of Medicine, University of Ottawa. J.W.S. was supported by the CIHR-AMGEN Canada Inc Chair in Kidney Research and support was in part provided by the CIHR. R.H. was supported by Banting and Best Diabetes Centre Graduate Studentships (University of Toronto), a Hilda and William Courtney Clayton Paediatric Research Fund Award and an Institute of Medical Science Graduate Student Award. Y.L. was supported by a Heart & Stroke/Richard Lewar Centre of Excellence Studentship, a Javenthey Soobiah Scholarship, Queen Elizabeth II/Dr. Arnie Aberman Scholarship in Science and Technology, a University of Toronto Fellowship in the Department of Pharmacology and Toxicology and a Canadian Diabetes Association Postdoctoral Fellowship. Finally, the authors are grateful to the study participants whose time and effort are critical to the success of our research program. D.Z.I.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

FUNDING SOURCES:

AdDIT is funded by the Juvenile Diabetes Research Foundation, British Heart Foundation and Diabetes UK. Funding for the Toronto center was also provided by the Juvenile Diabetes Research Foundation - Canadian Clinical Trial Network (JDRF-CCTN), the Canadian Diabetes Association, the Heart and Stroke Foundation of Canada and the SickKids Labatt Family Heart Center Innovation fund.

DISCLOSURES:

None

CONTRIBUTIONS:

KDB, YL, FHM, DD, LD, DBD, JD, NRD, YE, RH, JADV, TJB, CS, WH, FX, JZ, LM, RM, HNR, ES, JWS, DZIC, researched data, wrote the manuscript, contributed to discussion, reviewed/edited manuscript. All authors have approved the final version of this manuscript.

REFERENCES:

1. Adolescent type 1 Diabetes Cardio-renal Intervention Trial (AdDIT). *BMC Pediatr* 9: 79, 2009.
2. **Benter IF, Yousif MH, Cojocel C, Al-Maghrebi M, and Diz DI**. Angiotensin-(1-7) prevents diabetes-induced cardiovascular dysfunction. *Am J Physiol Heart Circ Physiol* 292: H666-672, 2007.
3. **Bernardi S, Burns WC, Toffoli B, Pickering R, Sakoda M, Tsorotes D, Grixti E, Velkoska E, Burrell LM, Johnston C, Thomas MC, Fabris B, and Tikellis C**. Angiotensin-converting enzyme 2 regulates renal atrial natriuretic peptide through angiotensin-(1-7). *Clin Sci (Lond)* 123: 29-37, 2012.
4. **Carey RM, and Siragy HM**. The intrarenal renin-angiotensin system and diabetic nephropathy. *Trends in endocrinology and metabolism: TEM* 14: 274-281, 2003.
5. **Cherney DZ, Perkins BA, Soleymanlou N, Xiao F, Zimpelmann J, Woerle HJ, Johansen OE, Broedl UC, von Eynatten M, and Burns KD**. Sodium glucose cotransport-2 inhibition and intrarenal RAS activity in people with type 1 diabetes. *Kidney international* 86: 1057-1058, 2014.
6. **Cherney DZ, Xiao F, Zimpelmann J, Har RL, Lai V, Scholey JW, Reich HN, and Burns KD**. Urinary ACE2 in healthy adults and patients with uncomplicated type 1 diabetes. *Canadian journal of physiology and pharmacology* 92: 703-706, 2014.
7. **Epstein MP, Lin X, and Boehnke M**. A tobit variance-component method for linkage analysis of censored trait data. *American journal of human genetics* 72: 611-620, 2003.
8. **Har RL, Reich HN, Scholey JW, Daneman D, Dunger DB, Moineddin R, Dalton RN, Motran L, Elia Y, Deda L, Ostrovsky M, Sochett EB, Mahmud FH, and Cherney DZ**. The urinary cytokine/chemokine signature of renal hyperfiltration in adolescents with type 1 diabetes. *PloS one* 9: e111131, 2014.
9. **Hsieh TJ, Fustier P, Wei CC, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Fantus IG, Hamet P, and Chan JS**. Reactive oxygen species blockade and action of insulin on expression of angiotensinogen gene in proximal tubular cells. *J Endocrinol* 183: 535-550, 2004.
10. **Hsieh TJ, Fustier P, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Fantus IG, Hamet P, and Chan JS**. High glucose stimulates angiotensinogen gene expression and cell hypertrophy via activation of the hexosamine biosynthesis pathway in rat kidney proximal tubular cells. *Endocrinology* 144: 4338-4349, 2003.
11. **Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, and Chan JS**. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. *Endocrinology* 143: 2975-2985, 2002.
12. **Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AH, Ishida Y, Inagami T, and Saruta T**. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. *The Journal of clinical investigation* 114: 1128-1135, 2004.
13. **Marcovecchio ML, Woodside J, Jones T, Daneman D, Neil A, Prevost T, Dalton RN, Deanfield J, and Dunger DB**. Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial (AdDIT): urinary screening and baseline biochemical and cardiovascular assessments. *Diabetes Care* 37: 805-813, 2014.
14. **Mizuiri S, Aoki T, Hemmi H, Arita M, Sakai K, and Aikawa A**. Urinary angiotensin-converting enzyme 2 in patients with CKD. *Nephrology (Carlton, Vic)* 16: 567-572, 2011.

15. **Mizuiri S, Hemmi H, Arita M, Ohashi Y, Tanaka Y, Miyagi M, Sakai K, Ishikawa Y, Shibuya K, Hase H, and Aikawa A**. Expression of ACE and ACE2 in individuals with diabetic kidney disease and healthy controls. *American journal of kidney diseases : the official journal of the National Kidney Foundation* 51: 613-623, 2008.
16. **Nadarajah R, Milagres R, Dilauro M, Gutsol A, Xiao F, Zimpelmann J, Kennedy C, Wysocki J, Batlle D, and Burns KD**. Podocyte-specific overexpression of human angiotensin-converting enzyme 2 attenuates diabetic nephropathy in mice. *Kidney Int* 82: 292-303, 2012.
17. **Nakatani S, Ishimura E, Naganuma T, Nakatani A, Ichii M, Fukumoto S, Mori K, Emoto M, Nakatani T, and Inaba M**. Poor glycemic control and decreased renal function are associated with increased intrarenal RAS activity in Type 2 diabetes mellitus. *Diabetes research and clinical practice* 105: 40-46, 2014.
18. **Odutayo A, and Cherney D**. Cystatin C and acute changes in glomerular filtration rate. *Clinical nephrology* 78: 64-75, 2012.
19. **Park SE, Kim WJ, Park SW, Park JW, Lee N, Park CY, and Youn BS**. High urinary ACE2 concentrations are associated with severity of glucose intolerance and microalbuminuria. *European journal of endocrinology / European Federation of Endocrine Societies* 168: 203-210, 2013.
20. **Perkins BA, Sochett EB, and Cherney DZ**. Ability of Cystatin C to detect changes in glomerular filtration rate after ACE inhibition in patients with uncomplicated type 1 diabetes. *Clinical and experimental hypertension (New York, NY : 1993)* 34: 606-611, 2012.
21. **Price DA, Porter LE, Gordon M, Fisher ND, De'Oliveira JM, Laffel LM, Passan DR, Williams GH, and Hollenberg NK**. The paradox of the low-renin state in diabetic nephropathy. *Journal of the American Society of Nephrology : JASN* 10: 2382-2391, 1999.
22. **Ramaha A, Celerier J, and Patston PA**. Characterization of different high molecular weight angiotensinogen forms. *Am J Hypertens* 16: 478-483, 2003.
23. **Saito T, Urushihara M, Kotani Y, Kagami S, and Kobori H**. Increased urinary angiotensinogen is precedent to increased urinary albumin in patients with type 1 diabetes. *The American journal of the medical sciences* 338: 478-480, 2009.
24. **Salem ES, Grobe N, and Elased KM**. Insulin treatment attenuates renal ADAM17 and ACE2 shedding in diabetic Akita mice. *Am J Physiol Renal Physiol* 306: F629-639, 2014.
25. **Satirapoj B, Siritaweesuk N, and Supasyndh O**. Urinary angiotensinogen as a potential biomarker of diabetic nephropathy. *Clinical kidney journal* 7: 354-360, 2014.
26. **Shi Y, Lo CS, Padda R, Abdo S, Chenier I, Filep JG, Ingelfinger JR, Zhang SL, and Chan JS**. Angiotensin-(1-7) prevents systemic hypertension, attenuates oxidative stress and tubulointerstitial fibrosis, and normalizes renal angiotensin-converting enzyme 2 and Mas receptor expression in diabetic mice. *Clinical science (London, England : 1979)* 128: 649-663, 2015.
27. **Shiota A, Yamamoto K, Ohishi M, Tatara Y, Ohnishi M, Maekawa Y, Iwamoto Y, Takeda M, and Rakugi H**. Loss of ACE2 accelerates time-dependent glomerular and tubulointerstitial damage in streptozotocin-induced diabetic mice. *Hypertension research : official journal of the Japanese Society of Hypertension* 33: 298-307, 2010.
28. **Soler MJ, Wysocki J, and Batlle D**. ACE2 alterations in kidney disease. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* 2013.

29. **Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y, and Batlle D**. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. *Kidney international* 72: 614-623, 2007.
30. **Soltysiak J, Skowronska B, Fichna P, Ostalska-Nowicka D, Stankiewicz W, Lewandowska-Stachowiak M, Lipkowska K, and Zachwieja J**. Urinary angiotensinogen and urinary sodium are associated with blood pressure in normoalbuminuric children with diabetes. *Pediatric nephrology (Berlin, Germany)* 29: 2373-2378, 2014.
31. **Soro-Paavonen A, Gordin D, Forsblom C, Rosengard-Barlund M, Waden J, Thorn L, Sandholm N, Thomas MC, and Groop PH**. Circulating ACE2 activity is increased in patients with type 1 diabetes and vascular complications. *J Hypertens* 30: 375-383, 2012.
32. **Tikellis C, Brown R, Head GA, Cooper ME, and Thomas MC**. Angiotensin-converting enzyme 2 mediates hyperfiltration associated with diabetes. *Am J Physiol Renal Physiol* 306: F773-780, 2014.
33. **Uri K, Fagyas M, Manyine Siket I, Kertesz A, Csanadi Z, Sandorfi G, Clemens M, Fedor R, Papp Z, Edes I, Toth A, and Lizanecz E**. New perspectives in the renin-angiotensin-aldosterone system (RAAS) IV: circulating ACE2 as a biomarker of systolic dysfunction in human hypertension and heart failure. *PLoS One* 9: e87845, 2014.
34. **Wang G, Lai FM, Lai KB, Chow KM, Kwan CH, Li KT, and Szeto CC**. Urinary mRNA expression of ACE and ACE2 in human type 2 diabetic nephropathy. *Diabetologia* 51: 1062-1067, 2008.
35. **Wang TT, Wu XH, Zhang SL, and Chan JS**. Effect of glucose on the expression of the angiotensinogen gene in opossum kidney cells. *Kidney international* 53: 312-319, 1998.
36. **Whaley-Connell AT, Chowdhury NA, Hayden MR, Stump CS, Habibi J, Wiedmeyer CE, Gallagher PE, Tallant EA, Cooper SA, Link CD, Ferrario C, and Sowers JR**. Oxidative stress and glomerular filtration barrier injury: role of the renin-angiotensin system in the Ren2 transgenic rat. *American journal of physiology Renal physiology* 291: F1308-1314, 2006.
37. **Wysocki J, Garcia-Halpin L, Ye M, Maier C, Sowers K, Burns KD, and Batlle D**. Regulation of urinary ACE2 in diabetic mice. *American journal of physiology Renal physiology* 305: F600-611, 2013.
38. **Wysocki J, Ortiz-Melo DI, Mattocks NK, Xu K, Prescott J, Evora K, Ye M, Sparks MA, Haque SK, Batlle D, and Gurley SB**. ACE2 deficiency increases NADPH-mediated oxidative stress in the kidney. *Physiol Rep* 2: e00264, 2014.
39. **Xiao F, Hiremath S, Knoll G, Zimpelmann J, Srivaratharajah K, Jadhav D, Fergusson D, Kennedy CR, and Burns KD**. Increased urinary angiotensin-converting enzyme 2 in renal transplant patients with diabetes. *PloS one* 7: e37649, 2012.
40. **Xiao F, Zimpelmann J, Agaybi S, Gurley SB, Puente L, and Burns KD**. Characterization of angiotensin-converting enzyme 2 ectodomain shedding from mouse proximal tubular cells. *PLoS One* 9: e85958, 2014.
41. **Ye M, Wysocki J, Naaz P, Salabat MR, LaPointe MS, and Batlle D**. Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination? *Hypertension* 43: 1120-1125, 2004.
42. **Ye M, Wysocki J, William J, Soler MJ, Cokic I, and Batlle D**. Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. *J Am Soc Nephrol* 17: 3067-3075, 2006.

587 **Table 1. Baseline demographic characteristics of healthy controls (HC) and type 1 diabetes**

588 **adolescent patients (T1D).**

Parameter	HC (n=65)	T1D (n=194)
Baseline demographic parameters		
Males	28 (43%)	97 (50%)
Age (years)	14.0±2.0	14.4±1.7
Diabetes duration (years)	-	7.2±3.1
Body mass index (z-score)	0.11±1.14	0.62±0.89[a]
Baseline biochemistry		
Hemoglobin A1c - % (mmol/mol)	5.4±0.2	8.5±1.2 [a]
	(35.3±2.7)	(69.0±13.4) [a]
Plasma Glucose (mM)	4.7±0.7	9.7±4.2 [a]
Cholesterol (mM)	4.2±0.8	4.3±0.9
HDL Cholesterol (mM)	1.5±0.3	1.6±0.4 [a]
LDL Cholesterol (mM)	2.4±0.7	2.3±0.7
Triglyceride (mM)	0.9±0.4	0.8±0.3
Renal function assessments		
eGFR (mL/min/1.73 m^2)	121±22	129±29
Urine ACR (mg/mmol)	1.1±1.6	1.0±1.5
Blood Pressure and Heart Rate		
HR (beats per minute)	69±11	67±8
SBP (mmHg)	111±8	114±9 [a]

DBP (mmHg)	63±5	63±6
SBP (z-score)	0.04±0.77	0.19±0.87
DBP (z-score)	-0.28±0.71	-0.26±0.64
Urinary RAAS Markers		
Angiotensinogen (ng/mg Cr)	0.7 (0.0-1.8)	2.5 (0.7-6.1) [a]
ACE Activity (ng/mg Cr)	0.0 (0.0-0.0)	0.4 (0.0-1.6) [a]
ACE2 Activity (ng/mg Cr)	0.0 (0.0-0.0)	79.1 (0.0-418.1) [a]
ACE2 (ng/mg Cr)	0.0 (0.0-1.3)	2.7 (0.7-13.1) [a]

n, number of participants. [a] $p<0.0125$ vs. HC; HC: healthy controls; T1D: type 1 diabetic patients; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; ACR: albumin to creatinine ratio; RAAS: renin-angiotensin aldosterone system; ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2.

Table 2. Liner regression analysis of log transformed angiotensinogen (A), log ACE Activity (B), log ACE2 Activity (C) and log ACE2 levels (D) with HbA1c and log transformed urine albumin to creatinine ratio (ACR) in patients with type 1 diabetes (T1D, n=194).

(A)

	Log Angiotensinogen			
	Regression		TOBIT	
	β	p	β	p
Blood Glucose	0.01	NS	0.02	NS
HbA1c	0.11	<0.0001	0.14	<0.0001
logACR	0.44	<0.0001	0.50	<0.0001

(B)

	Log ACE Activity			
	Regression		TOBIT	
	β	p	β	p
Blood Glucose	0.02	<0.0001	0.03	<0.0001
HbA1c	0.04	0.011	0.80	0.003
logACR	0.13	NS	0.26	0.007

(C)

	Log ACE2 Activity			
	Regression		TOBIT	
	β	p	β	p
Blood Glucose	0.04	NS	0.07	NS
HbA1c	0.21	0.003	0.31	0.007
logACR	0.61	NS	0.84	NS

(D)

	Log ACE2			
	Regression		TOBIT	
	β	**p**	**β**	**p**
Blood Glucose	0.02	NS	0.02	NS
HbA1c	0.11	0.0004	0.13	0.0003
logACR	0.32	0.005	0.30	NS

608 NS = not statistically significant. The β coefficients with the associated p value were obtained
609 from the regression analysis. The adjusted TOBIT analysis was performed to further adjust for
610 below detection levels of respective RAAS markers, age, gender, BMI z score, T1D duration and
611 HDL cholesterol(7).

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Table 3: Spearman correlation coefficients (r) and significance levels (p) of associations between urinary RAAS markers and systemic vascular function in adolescents with type 1 diabetes vs. healthy controls.

	Angiotensinogen				ACE Activity				ACE2 Activity				ACE2			
	HC		T1D		HC		T1D		HC		T1D		HC		T1D	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
SBP (z-score)	0.11	0.36	-0.040	0.58	0.09	0.49	0.0007	0.99	0.10	0.41	-0.009	0.90	-0.02	0.89	-0.02	0.74
DBP (z-score)	-0.05	0.69	-0.11	0.12	0.08	0.53	0.13	0.08	0.06	0.61	0.10	0.16	0.05	0.71	0.10	0.16
HR	0.08	0.52	0.09	0.20	0.22	0.08	0.080	0.27	0.17	0.17	0.03	0.73	0.08	0.54	0.015	0.83

HC: healthy controls; T1D: type 1 diabetic patients; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2.

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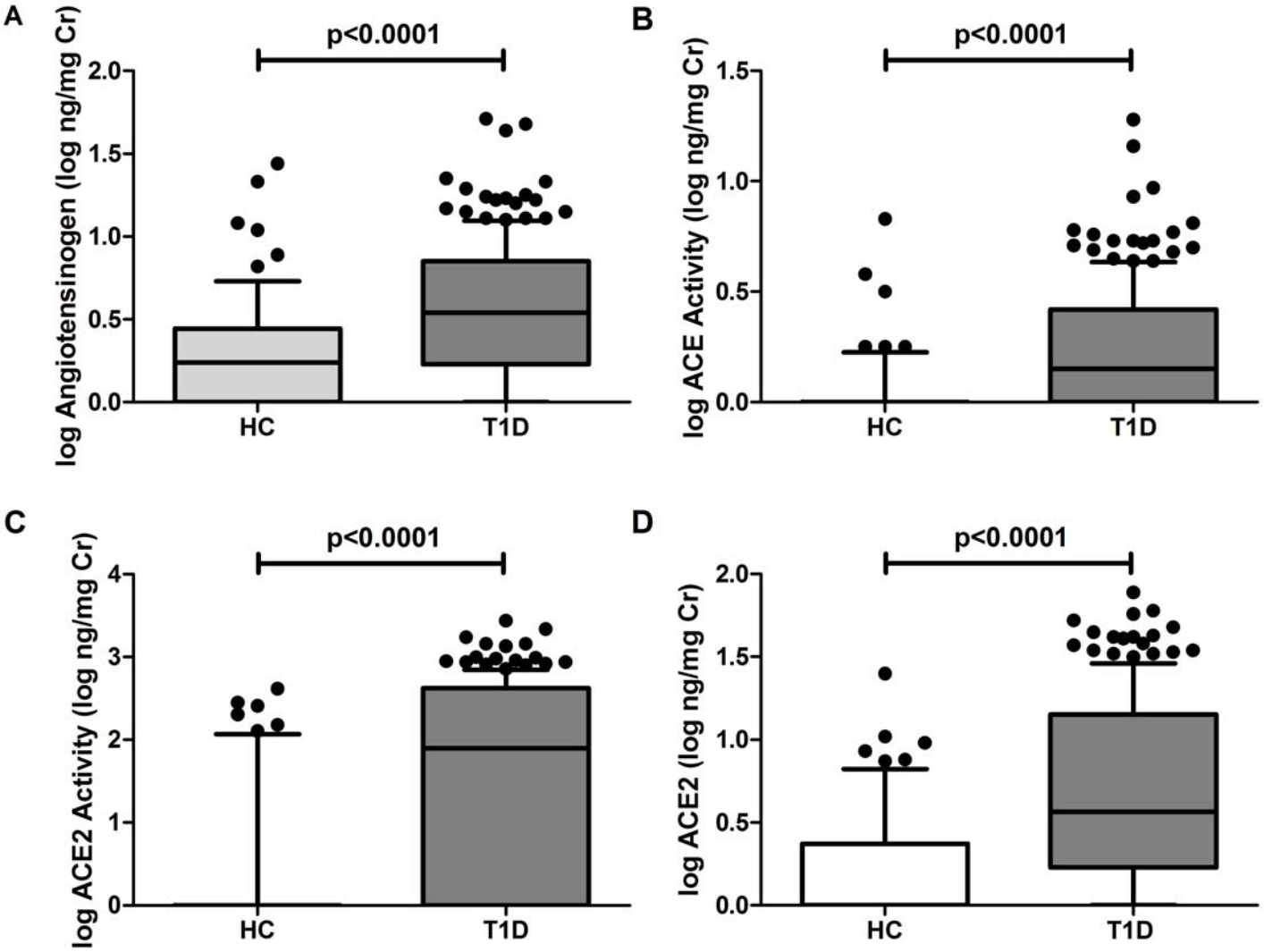
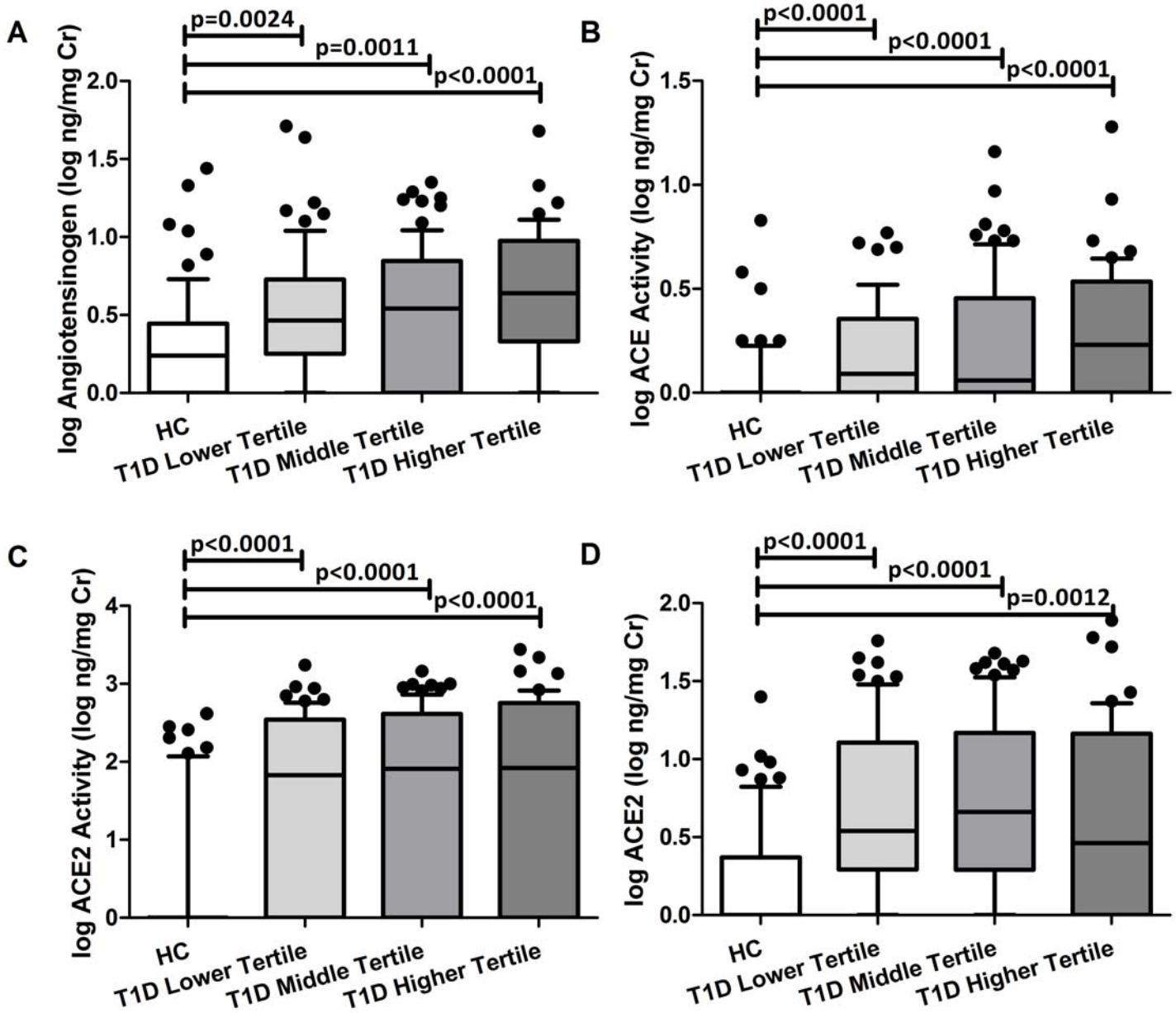
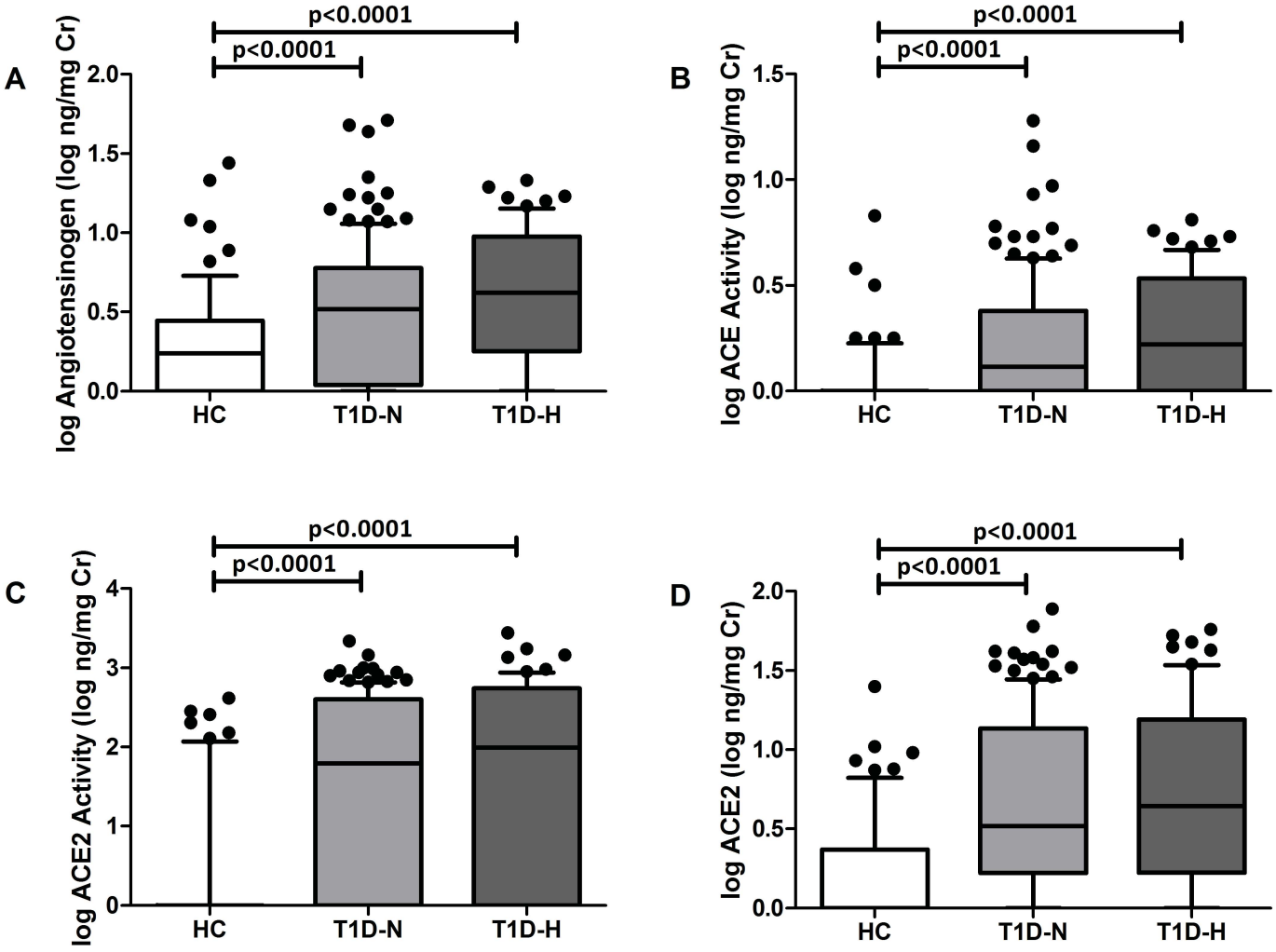
Figure 1. Urinary Angiotensinogen (A), ACE Activity (B), ACE2 Activity (C) and ACE2 levels (D) in healthy controls (HC, n=65) and patients with type 1 diabetes (T1D, n=194). Log transformed data are represented as median, interquartile range and 10^{th} to 90^{th} percentile.

Figure 2: Angiotensinogen (A), ACE Activity (B), ACE2 Activity (C) and ACE2 levels (D) levels in HC (n=65) and patients with type 1 diabetes (T1D) in the low ACR tertile (<0.8mg/mmol, n=64), middle ACR tertile (0.8-1.2mg/mmol, n=77) and high ACR tertile (>1.2mg/mmol, n=53). Log transformed data are represented as median, interquartile range and 10^{th} to 90^{th} percentile.

Figure 3: Angiotensinogen (A), ACE Activity (B), ACE2 Activity (C) and ACE2 levels (D) levels in HC (n=65) and patients with type 1 diabetes (T1D) with normofiltration (T1D-N, GFR<135mL/min/1.73m^2, n=132) and hyperfiltration (T1D-H, GFR≥135mL/min/1.73m^2, n= 62). Log transformed data are represented as median, interquartile range and 10^{th} to 90^{th} percentile.


A
log Angiotensinogen (log ng/mg Cr)
p<0.0001
2.0
1.5
1.0
0.5
0.0
HC
T1D
B
log ACE Activity (log ng/mg Cr)
p<0.0001
1.5
1.0
0.5
0.0
HC
T1D
C
log ACE2 Activity (log ng/mg Cr)
p<0.0001
4
3
2
1
0
HC
T1D
D
log ACE2 (log ng/mg Cr)
p<0.0001
2.0
1.5
1.0
0.5
0.0
HC
T1D


A
p=0.0024
p=0.0011
p<0.0001
log Angiotensinogen (log ng/mg Cr)
2.0
1.5
1.0
0.5
0.0
HC
T1D Lower Tertile
T1D Middle Tertile
T1D Higher Tertile
B
p<0.0001
p<0.0001
p<0.0001
log ACE Activity (log ng/mg Cr)
1.5
1.0
0.5
0.0
HC
T1D Lower Tertile
T1D Middle Tertile
T1D Higher Tertile
C
p<0.0001
p<0.0001
p<0.0001
log ACE2 Activity (log ng/mg Cr)
4
3
2
1
0
HC
T1D Lower Tertile
T1D Middle Tertile
T1D Higher Tertile
D
p<0.0001
p<0.0001
p=0.0012
log ACE2 (log ng/mg Cr)
2.0
1.5
1.0
0.5
0.0
HC
T1D Lower Tertile
T1D Middle Tertile
T1D Higher Tertile


A
p<0.0001
p<0.0001
log Angiotensinogen (log ng/mg Cr)
2.0
1.5
1.0
0.5
0.0
HC
T1D-N
T1D-H
B
p<0.0001
p<0.0001
log ACE Activity (log ng/mg Cr)
1.5
1.0
0.5
0.0
HC
T1D-N
T1D-H
C
p<0.0001
p<0.0001
log ACE2 Activity (log ng/mg Cr)
4
3
2
1
0
HC
T1D-N
T1D-H
D
p<0.0001
p<0.0001
log ACE2 (log ng/mg Cr)
2.0
1.5
1.0
0.5
0.0
HC
T1D-N
T1D-H