Paper Title

Enhanced angiotensinogen expression in neonates during kidney development

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Concise Title

Angiotensinogen expression in neonates

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Abstract

Background

We recently demonstrated that preterm neonates have higher urinary angiotensinogen (AGT) levels than full-term neonates. Here, we tested the hypothesis that enhanced neonatal AGT expression is associated with intrarenal renin-angiotensin system (RAS) status during kidney development.

Methods

We prospectively recruited neonates born at our hospital and healthy children with minor glomerular abnormalities between April 2013 and March 2017. We measured neonatal plasma and urinary AGT levels at birth and one year later and assessed renal AGT expression in kidney tissues from neonates and healthy children using immunohistochemical (IHC) analysis.

Results

Fifty-four neonates and eight children were enrolled. Although there were no changes in plasma AGT levels, urinary AGT levels were significantly decreased one year after birth. Urinary AGT levels at birth were inversely correlated with gestational age, and urinary AGT levels at birth and one year later were inversely correlated with estimated glomerular filtration rate one year after birth. IHC analysis showed that renal AGT expression in neonates was higher than that in healthy children and inversely correlated with gestational age.

Conclusions

Enhanced AGT expression and urinary AGT excretion may reflect intrarenal RAS activation associated with kidney development in utero.

Key words

Angiotensinogen, renin-angiotensin system, kidney development, neonates

Introduction

The renin-angiotensin system (RAS) plays central roles in blood pressure regulation and the maintenance of renal hemodynamics, sodium transport, and glomerular filtration in both normal physiological states and pathological conditions [1, 2]. Angiotensin II (Ang II) is the most powerful biologically active product of the RAS and derives from its locally formed substrate, angiotensinogen (AGT), in the kidney [3]. AGT is the only known substrate for renin, a rate-limiting enzyme of the RAS. Experimental studies have demonstrated that AGT levels in renal tissues reflect the activity of the intrarenal RAS [4] [2]. A direct quantitative method to measure urinary AGT using human AGT enzyme-linked immunosorbent assays (ELISA) was developed [5] that revealed significantly increased urinary AGT levels in patients with hypertension, chronic kidney disease, diabetes, and acute kidney injury [2] [6].

Recent studies have demonstrated the contribution of RAS to the mammalian kidney development [7] [8]. Many studies established the importance of an intact RAS cascade during renal development using both pharmacological inhibition and genetic deletions affecting various RAS components. Prenatal insults likely result in renal injury by disturbing the RAS as well as other factors necessary for normal kidney development [8].

Our previous study demonstrated that urinary AGT levels at birth are significantly higher in preterm neonates than in full-term neonates, suggesting intrarenal RAS activation during kidney development [9]. Another study reported that urinary AGT could be an efficient tool for screening for future renal dysfunction [10]. In the present study, we measured the urinary and plasma levels of AGT at birth and one year later and investigated the expression of AGT in neonatal kidney tissues to test the hypothesis that urinary AGT is associated with renal development and function in human infants.

Material and Methods

Patients and samples

The Institutional Review Board of Tokushima University approved the study protocol and the use of tissue samples. Informed consent was obtained from the parents of each enrolled neonate. We prospectively recruited study participants at Tokushima University Hospital between April 1, 2013, and March 31, 2017. Our exclusion criteria included suspected sepsis, severe respiratory distress syndrome, congenital heart disease, and renal or chromosomal abnormalities. Neonates expected to die within 48 hours of recruitment were also excluded. We recorded demographic and perinatal characteristics including gestational age (GA), birth weight, sex, and Apgar scores at 1 and 5 minutes for all neonates. Blood and urine samples were collected within days after birth and one year later. The samples were stored at -20 °C until biochemical analysis. Tissue samples were obtained at the time of autopsy in newborns that died of pulmonary hypoplasia. We also recruited eight participants, 2–8 years of age, with minor glomerular abnormalities resulting in mild proteinuria or microscopic hematuria in spite of normal glomerular morphology and negative immunofluorescence on renal biopsy examination.

Measurements

Urinary creatinine concentration was measured using the Creatinine Assay Kit (BioVision, Inc., Milpitas, CA, US). Plasma and urinary concentrations of AGT were measured using commercially available ELISA kits (Immuno-Biological Laboratories Co, Ltd. [IBL], Gunma, Japan), as previously described [11]. The sensitivity of this assay is > 0.31 ng/mL. The intra- and inter-assay coefficients of variation were 4.4 and 4.3%, respectively. The urinary level of AGT was expressed as the AGT/creatinine ratio. The estimated glomerular filtration rate (eGFR) one year after birth was calculated using the formula developed by Uemura et al.

(104.1 x 1/serum cystatin C - 7.80) [12].

Immunohistochemistry

The tissues obtained at the time of autopsy were fixed in 10% buffered formalin and embedded in paraffin. The paraffin sections (3-μm thick) were incubated overnight at 4 °C with an anti-AGT antibody (405, IBL) or without the primary antibody, then rinsed and incubated with the biotinylated secondary antibody (Vector Labs Inc, Burlingame, CA, US). After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC Elite, Vecter Labs Inc) followed by 3,3'-diaminobenzidine (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Each section was counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries, Ltd. Osaka, Japan), dehydrated, and cover-slipped. Quantification was performed using the EIS Elements software (Nikon Corporation, Tokyo, Japan). The immunoreactive area (brown) was assessed by setting a threshold and automatically calculated in arbitrary units. We measured AGT-positive area on cortical regions containing glomerulus and ten non-overlapping images per neonate were randomly selected and captured in a blinded manner. The integer optical density of the positive brown staining was determined.

Statistical analysis

Pearson and Spearman correlation coefficients were used for parametric and nonparametric data, respectively. The Wilcoxon signed-rank test was used to perform paired comparisons at birth and one year later. All data are presented as mean ± standard error of the mean (SEM). A $P < 0.05$ was considered significant. All computations, including data management and statistical analysis, were performed with JMP software (SAS Institute, Cary, NC, US) and GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, US).

Results

Subject profiles

The profiles of the study participants are summarized in Table 1. Fifty-four neonates were recruited, comprising 21 females and 33 males. The average GA of neonates was 32.06 ± 0.59 weeks, and the average weight was 1,531 ± 71.95 g. The average Apgar scores at 1 and 5 min were 6.19 ± 0.35 and 8.98 ± 0.22, respectively.

Plasma and urinary AGT levels

Figure 1 illustrates plasma AGT levels and urinary AGT/creatinine ratios. While no significant change in plasma AGT occurred in the first year of life, the urinary AGT/creatinine ratio significantly decreased in the one-year interval after birth.

Single regression analysis

Urinary AGT/creatinine ratios at birth were significantly and inversely correlated with GA, while those measured one year later showed no correlation with GA. Plasma AGT levels were not associated with GA at either time (Table 2, Figure 2). Urinary AGT/creatinine ratios at birth and one year later were significantly and inversely correlated with cystatin C-based eGFR measured one year after birth. However, plasma AGT levels at birth and one year later were not associated with cystatin C-based eGFR one year after birth (Table 3).

Multiple regression analysis

Factors showing a significant correlation in single regression analysis were adopted as

explanatory variables in multiple regression analysis (Table 4). As shown in Figure 3, urinary AGT/creatinine ratios at birth and after one year accounted for 22.68% of the variation in the cystatin C-based eGFR measured one year after birth ($r = 0.4762$, $R2 = 0.2268$, $P = 0.0031$).

Renal AGT expression in neonates

We next examined AGT expression in kidney tissues from neonates and children with minor glomerular abnormalities. AGT mainly localized in the proximal tubules in neonates. On the other hand, positive, but weak, AGT staining was observed in the proximal tubules with minor glomerular abnormalities. Renal AGT expression was significantly increased in neonates compared with that seen in children with minor glomerular abnormalities (Figure 4). In addition, the level of AGT expression in neonate kidneys was significantly and inversely correlated with GA (Figure 4).

Discussion

Recent studies demonstrated that urinary AGT levels are involved in RAS activation and the development of cardiovascular and kidney disease [2] [6]. Studies in human have established a link between urinary AGT and kidney development [6]. However, urinary AGT excretion and the renal AGT expression associated with kidney injury pathophysiology have not been studied extensively in humans, especially in neonates. Our study is the first to demonstrate that urinary AGT decreases during the first year of life. Furthermore, renal AGT expression was significantly higher in neonates than in children. In addition, urinary AGT levels at birth and one year later were inversely correlated with cystatin C-based eGFR measured one year after birth.

All the components of the RAS are present in the fetus where it plays a vital role in the development of the kidney [13] [8]. The circulating renin and Ang II levels during fetal life

are higher compared to during postnatal life [14]. Component production is precisely time-regulated, suggesting that Ang II could also exert its effects as a growth-promoting agent during kidney development [15]. Furthermore, a number of studies have examined the effect of prenatal programming on the intrarenal RAS. All components of the RAS are expressed in the kidney as early as the fifth weeks of gestation in human embryos [14] [16]. During the gestation, the RAS of the fetal lamb responds to the same stimuli, such as blood volume depletion, furosemide, hypoxemia, and RAS blockade [17] [18]. Similarly, human fetuses exposed in to RAS blockers in utero are severely hypotensive at birth and sometimes develop irreversible renal lesions resulting in renal failure and anuria [19, 20]. On the other hand, inappropriate activation of the RAS during fetal life may have deleterious consequences [21] [14]. Thus, RAS plays an important role in kidney development, and disruption of the RAS to significant kidney developmental abnormalities [8].

Various studies have demonstrated increased urinary AGT levels not only in hypertensive subjects not under treatment with RAS blockers or inhibitors but also in patients with chronic kidney disease and urinary AGT can be used as an index of intrarenal RAS activation and thus may be a useful biomarker [3]. We previously tested the hypothesis that urinary AGT levels reflect intrarenal RAS status in pediatric chronic glomerulonephritis [11]. We found that urinary AGT levels were significantly increased in patients with chronic glomerulonephritis who were not treated with RAS blockers compared with levels in control individuals. Notably, patients with glomerulonephritis treated with RAS blockers showed a marked attenuation of this augmentation. Thus, the efficacy of RAS blockade in reducing intrarenal RAS activity can be confirmed by urinary AGT measurements in patients with chronic glomerulonephritis [11]. Also, we demonstrated that urinary AGT levels as well as renal AGT expression were decreased in pediatric IgA nephropathy patients treated with RAS blockade [22]. These findings suggest that urinary AGT can be a novel biomarker of

intrarenal RAS activation in pediatric patients. Furthermore, our previous observational study demonstrated that urinary AGT level is higher in preterm neonates than in full-term neonates and inversely correlated with GA. These findings in addition to the results of the present study suggest that urinary AGT levels may reflect intrarenal RAS activation associated with kidney development in neonates.

All components of the RAS are highly expressed in the developing kidney in a pattern, suggesting a role for RAS in renal development [23]. During fetal life, renin is detected in the developing renal artery, interlobar arteries, and arcuate arteries of the metanephric kidney [24]. AGT was detected in the proximal portion of the primitive tubules where it was macroscopically visible only as faint labeling and was expressed in the metanephric proximal tubules throughout gestation [16]. Ang II receptors are widely distributed in the mammalian fetus and are detected in early gestation [25]. Although Ang II might be maternally derived, the presence of Ang II receptors is a prerequisite condition for the target cells Ang II-stimulus response that allows this agent a functional role in fetal development. Indeed, in this study, we demonstrated that renal proximal tubule AGT expression is enhanced in neonates. Additionally, we found an inverse correlation between AGT expression in neonate kidney samples and GA. Although the number of specimens available to us was small and pulmonary hypoplasia by cardiorespiratory failure had any influences on AGT expression during neonatal intrarenal RAS activation, these findings reveal the physiological significance of AGT for intrarenal RAS activation during kidney development. It has been difficult to dissect the specific contribution of the circulation versus the local RAS to the regulation of renal function because components of the RAS in the proximal tubules can be absorbed or taken up by endocytosis as well as synthetized locally [26]. The precise contribution of intrarenal RAS to renal pathophysiology and development as compared with that of systemic RS needs to be determined by functional studies [27]. Recently, we reported that (pro)rein receptor may play

a pivotal role in prenatal kidney development in humans [28]. Future investigation would evaluate the role of (pro)renin receptor on AGT expression in neonatal kidney.

Small size for GA, prematurity, and low birth weight are associated with reduced nephron numbers and an increased risk of hypertension and kidney disease in later life [29]. Estimated and measured GFRs are often reduced in newborns and older children that exhibited prematurity, small GA, low birth weight, or a combination of these [29]. A large proportion of variability in nephron number is already apparent at birth and individuals born with a total nephron number towards the lower end of the range may be more susceptible to developing renal dysfunction [30] [31]. A reduced number of nephrons as a result of premature birth is associated with renal dysfunction in later life [29]. Furthermore, Nishizaki et al reported that urinary AGT levels are increased prior to the increase in hematuria, proteinuria, albuminuria, and urinary β2-microglobulin in very low-birth weight children after discharge from the neonatal intensive care unit, and eGFR and urinary AGT level are negatively correlated [10]. In the present study, we clarified that urinary AGT levels at birth and one year later were inversely correlated with cystatin C-based eGFR. These results added to those of our previous investigations, suggest that neonatal urinary AGT is a prognostically significant biomarker of renal dysfunction in later life.

The relatively small number of subjects in this study is a potential limitation that could restrict our ability to draw causal conclusions. However, our observations indicate that urinary AGT levels are increased, and renal AGT expression is enhanced in neonates. These data strongly support the hypothesis that the RAS plays an important role in nephrogenesis that is associated with increased AGT expression in the kidney. Furthermore, measuring urinary AGT levels in neonates might be a useful method to predict kidney development and function in later life. We emphasize that the present study is the first report of the novelty for urinary AGT at birth and one year later as a biomarker of intrarenal RAS activation and renal AGT

expression in human neonates. This pilot study provides new insights into the understanding of human kidney development that require further prospective analyses in large multicenter studies.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Human and animal rights

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee at which the studies were conducted (IRB approval number 1425) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figure Legends

Figure1.

Changes in plasma angiotensinogen (AGT) in neonates at one year after birth (A). Changes in urinary AGT/urinary creatinine ratio (UAGT/UCre) in neonates one year after birth (B). Each marker and line represents an individual study participant.

Figure 2.

Single regression analyses for urinary AGT-to-creatinine ratio (urinary AGT/Cre) with gestational age at birth (A) and one year after birth (B). The urinary AGT/Cre at birth showed inversely correlation with gestational age, but urinary AGT/Cre one year after birth did not. Two curves (dashed) surrounding linear regression line (solid) define the 95% confidence interval.

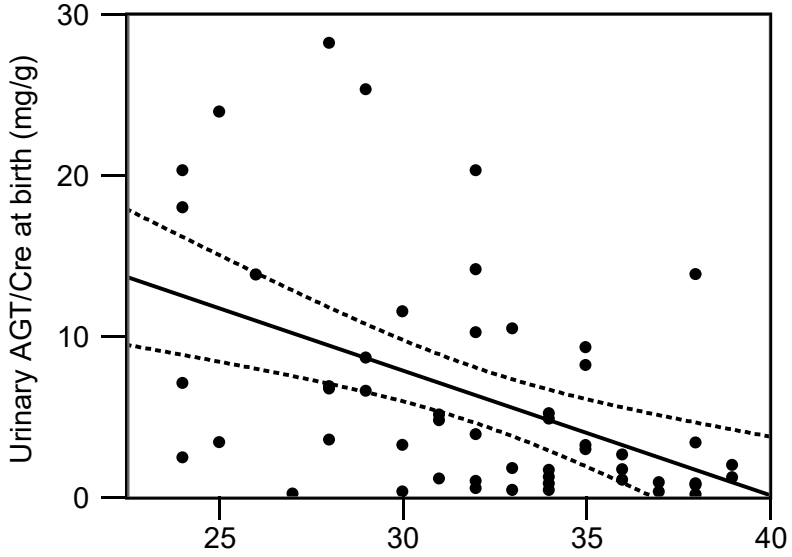
Figure 3.

Multiple-regression analysis of cystatin C-based estimated glomerular filtration ratio (eGFR) at one year after birth. Two parameters (urinary AGT/urinary creatinine ratio at birth and one year later) accounted for 22.68% of the variation in the cystatin C-based eGFR ($r = 0.4762$, $R^2 = 0.2268$, $P = 0.0031$).

Figure 4.

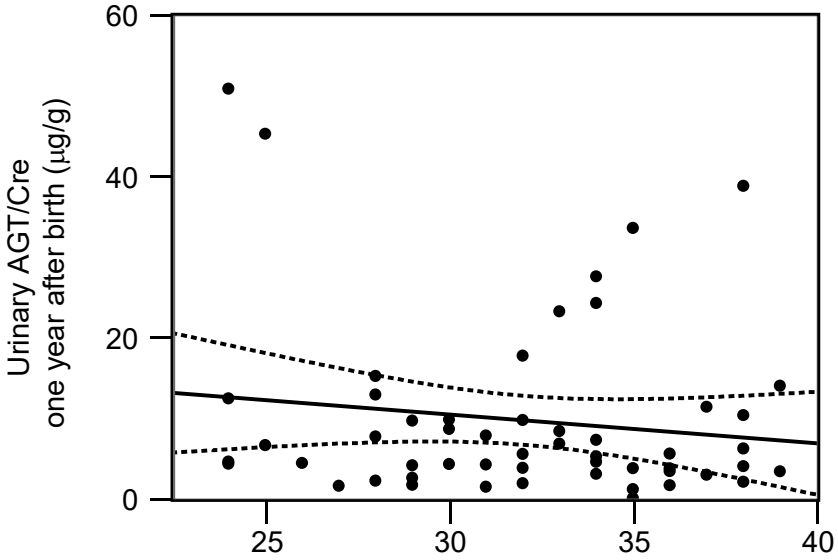
Renal tissue angiotensinogen (AGT) immunoreactivity in neonates with minor glomerular abnormalities. (A) Representative images of AGT immunostaining in a 29-week gestation neonates (a), 36- week gestation neonate (b), 2-year old child (c), and 8-year old child with minor glomerular abnormalities (d), negative control (e). Original magnification x400. (B) AGT levels in renal tissues are expressed in arbiter units (AU). (C) Single regression analysis of AGT expression levels in neonates renal in function of GA.

A



Urinary AGT/Cre = 31.01 – 0.77 Gestational age
r = -0.4714, P = 0.0003

B



Urinary AGT = 26.39 - 0.45 Gestational age
r = -0.1390, P = 0.3161

Figure 2

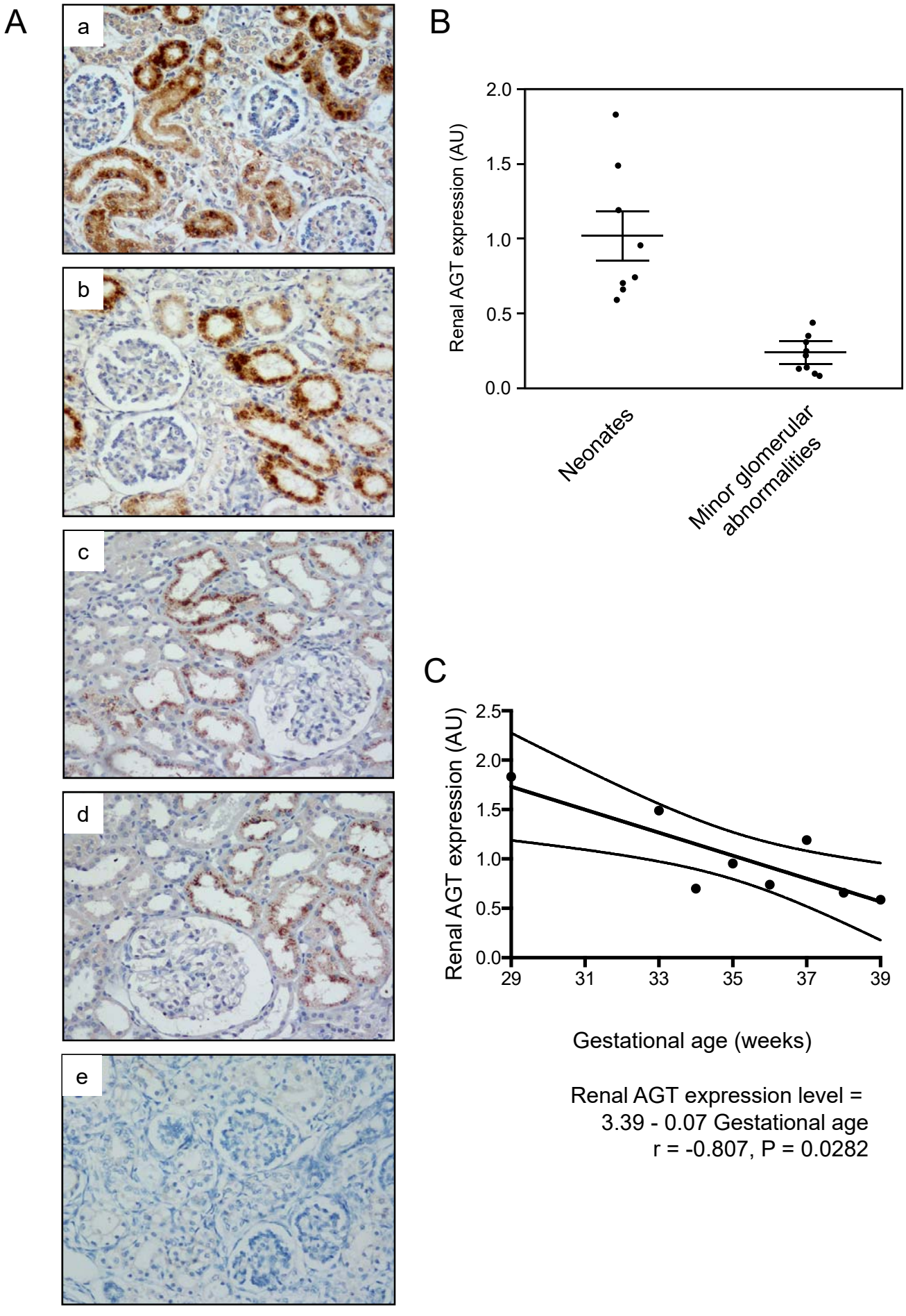
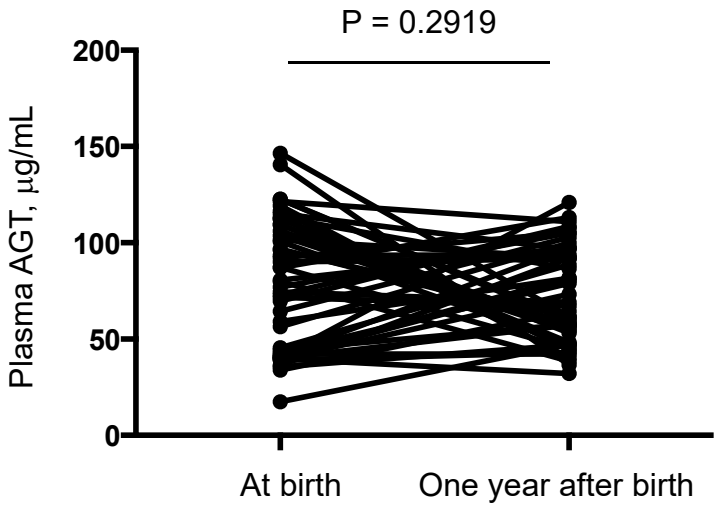

A
a
b
c
d
e
B
Renal AGT expression (AU)
2.0
1.5
1.0
0.5
0.0
Neonates
Minor glomerular abnormalities
C
Renal AGT expression (AU)
2.5
2.0
1.5
1.0
0.5
0.0
29
31
33
35
37
39
Gestational age (weeks)
Renal AGT expression level =
3.39 - 0.07 Gestational age
r = -0.807, P = 0.0282

Figure 4

A


P = 0.2919
200
150
100
50
0
Plasma AGT, µg/mL
At birth
One year after birth

B

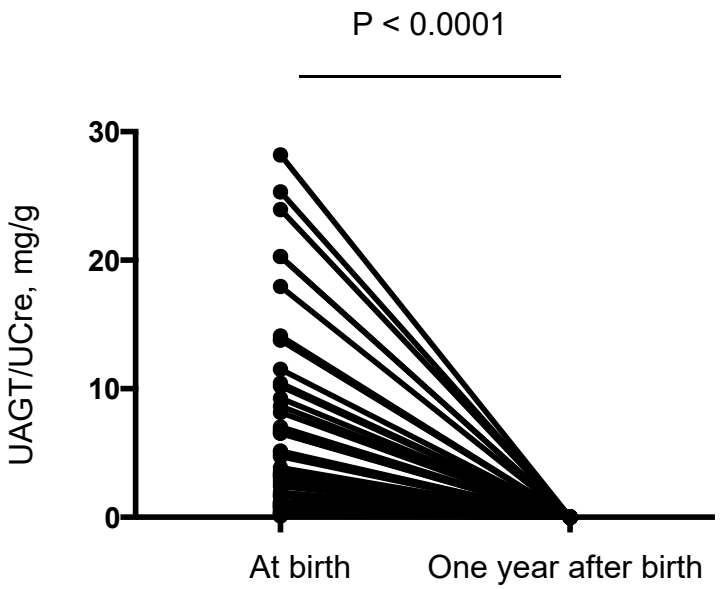
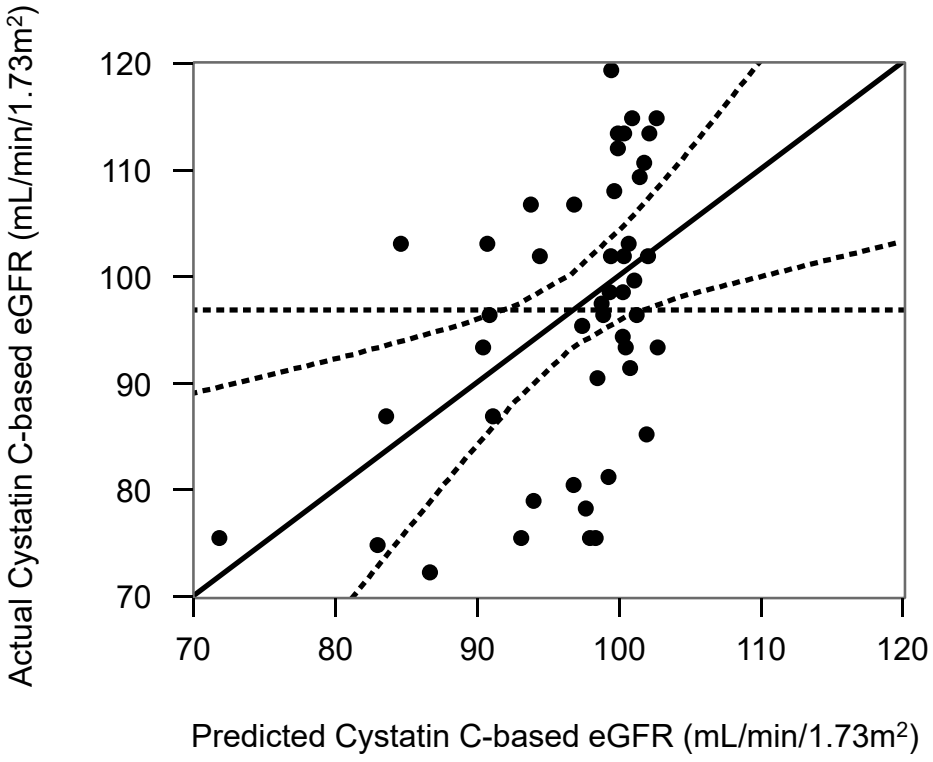

P < 0.0001
30
20
10
0
UAGT/UCre, mg/g
At birth
One year after birth

Figure 1



Cystatin C-based eGFR = 103.95 – 0.0006 UAGT/UCre at birth
- 0.31 UAGT/UCre one year after birth
r = 0.4762, p = 0.0031

Figure 3

Table 2. Gestational age and correlation

	Plasma AGT		Urinary AGT/UCre	
	R value	P values	R value	P values
At birth	-0.0467	0.7374	-0.4714	0.0003 *
One year after birth	-0.0019	0.9893	-0.1390	0.3161

UAGT/UCre; urinary angiotensinogen-creatinine ratio, *; $P < 0.05$, **; $P < 0.01$.

Table 3. Cystatin C-based eGFR and correlation

	Plasma AGT		Urinary AGT/UCre	
	R value	P values	R value	P values
At birth	-0.1781	0.1178	-0.3802	0.0077 **
One year after birth	0.0483	0.7440	-0.3409	0.0177 *

UAGT/UCre; urinary angiotensinogen-creatinine ratio, *; $P < 0.05$, **; $P < 0.01$.

Table 4. Cystatin C-based eGFR and correlation

Parameters	Estimate	SE	T ratio	P values
Intercept	103.96	2.62	39.61	< 0.0001 *
UAGT/UCre at birth, μg/g	-0.00	0.00	-2.54	0.0147 *
UAGT/UCre one year after birth, μg/g	-0.31	0.14	-2.19	0.0339 *

UAGT/UCre; urinary angiotensinogen-creatinine ratio, *; $P < 0.05$, **; $P < 0.01$.

Table 1. Subject profiles

Parameters	Data
Gestational age, weeks	32.1 +/- 0.6
Birth weight, g	1,531 +/- 72
Gender, F/M	21/33
Apgar score, 1 min	6.2 +/- 0.4
Apgar score, 5 min	9.0 +/- 0.2

F; Females, M; Males,