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Title: Increased podocyturia in pregnant women compared to non-pregnant women

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Abstract

Aim: Hyperfiltration is a cause of podocyturia and occurs physiologically in the kidney of pregnant women. Podocyturia is increased in preeclamptic pregnancies, but it is unclear whether there is also any increase in uncomplicated pregnancies. This study was performed to examine whether podocyturia and urine aquaporin 2 mRNA expression are increased in healthy pregnant women (PW) compared to healthy non-pregnant women (NPW).

Methods: Eleven urines obtained from 11 NPW and longitudinal 76 urines from 40 PW with uncomplicated pregnancies (median number [range] of urine samples/person, 2 [1 – 3]) were studied. Determination of protein and creatinine concentrations and number of cells in urine other than blood cells, and quantitative analyses of the mRNA expression of aquaporin 2 (AQP2-mRNA), podocin (Pod-mRNA), and nephrin (Nep-mRNA) were performed using RT-PCR in pelleted urine samples. Podocyturia was monitored with urine Pod- and Nep-mRNA expression levels normalized relative to creatinine.

Results: Urine cell density and urine AQP2-, Pod-, and Nep-mRNA expression normalized relative to creatinine were significantly higher in PW than NPW. The number of cells per milligram of creatinine was significantly positively correlated with expression of all three mRNAs with correlation coefficients (R-value) of 0.442, 0.481, and 0.561 for Pod-, Nep-, and AQP2-mRNA, respectively. AQP2-mRNA expression was strongly ($R > 0.8$) positively correlated with both Pod- and Nep-mRNA expression.

Conclusion: Podocyturia monitored by Pod- and Nep-mRNA expression and urine cells expressing AQP2-mRNA were increased in uncomplicated pregnancies compared to healthy non-pregnant women. Urine cells expressing AQP2-mRNA increased with increasing podocyturia in healthy women.

Key words: aquaporin, podocin, nephrin, podocyte injury, proteinuric diseases

INTRODUCTION

The podocytes are glomerular epithelial cells, located at the outermost layer of the glomerular basement membrane, the foot processes of which form tight interdigitating networks that regulate the filtration of circulating plasma proteins from the capillary lumen into Bowman's space [1, 2]. The podocytes detach from the glomerular basement membrane under various stimuli including hyperfiltration and hypertension [3], and are excreted in the urine, resulting in podocyturia [4]. Podocytes are present in the urine of healthy subjects without renal disease, although the number of urine podocytes is small, i.e., less than one podocyte per milligram of creatinine vs. up to 388 podocytes per milligram of creatinine in patients with active glomerular disease [4]. Increased podocyturia occurs in preeclamptic pregnancies compared to uncomplicated pregnancies [5 – 8]. Hyperfiltration occurs physiologically in response to increased plasma volume in pregnancy [9]. However, it is unclear whether podocyturia is increased in uncomplicated pregnancies compared to the non-pregnant state, possibly due to the lesser degree of podocyturia in both conditions.

Aquaporin 2 (AQP2), expressed in the principal cells of the kidney connecting tubule and collecting duct, but not in podocytes, plays a critical role in regulation of body water balance [10]. AQP2-mRNA and podocyte-specific protein mRNAs, including podocin (Pod-mRNA) and nephrin (Nep-mRNA), can be detected in the urine of otherwise healthy human subjects [11]. Pod-mRNA and Nep-mRNA expression are seen in urine of women with uncomplicated pregnancies [6, 8] and a low degree of podocyturia is monitored by urine Pod-mRNA and Nep-mRNA expression [6, 8]. However, it is unknown whether urine AQP2-mRNA expression level is increased in uncomplicated pregnancies compared to that in non-pregnant state.

The present study was performed to answer questions whether podocyturia and number of urine cells expressing AQP2-mRNA were increased in healthy women with uncomplicated pregnancies compared to those in otherwise healthy non-pregnant women.

METHODS

This study was conducted in accordance with the principles of the Declaration of Helsinki and with the approval of the Institutional Review Board of Hokkaido University Hospital (013-3999, April 30, 2014).

Eleven healthy non-pregnant women (NPW) and 40 healthy pregnant women (PW) with uncomplicated pregnancies participated in this study after giving written informed consent (Table 1). All 40 PW fulfilled the following two conditions: 1) normal blood pressure (defined as systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg) and no proteinuria (defined as spot urine protein:creatinine ratio [mg/mg] <

0.27) throughout pregnancy; and 2) gave birth at Hokkaido University Hospital during the study period from May 2014 to March 2016. Thus, no pregnant women with hypertensive disorders of pregnancy defined by the Japan Society for the Study of Hypertension in Pregnancy [12] were included in this study. The 11 NPW provided 11 urine samples and 40 PW provided 76 urine samples; 12 provided two urine samples (three women provided 1st and 2nd trimester urine samples, seven provided 2nd and 3rd trimester urine samples, and two provided 1st and 3rd trimester urine samples), 12 provided three urine samples collected at each trimester, and the remaining 16 women provided one urine sample (five, six, and five provided 1st, 2nd, and 3rd trimester urine samples, respectively). There were no differences in age, height, body weight, or body mass index (BMI) between the two groups (Table 1).

All spot urine samples were coded and processed within 2 hours of collection. Urine samples were centrifuged at $700 \times g$ for 5 minutes. Urinary supernatant was stored at -20°C until measurement of protein and creatinine (Cr) levels. The total number of cells other than blood cells (after excluding leukocytes and erythrocytes) in the pelleted urine stained with trypan blue was counted in five low-power ($\times 100$) visual fields by microscopy. The mean cell count per visual field was calculated and expressed as cell density per milligram of Cr. The pelleted urine samples were suspended in RNeasy lysis buffer (Life Technologies, Carlsbad, CA) and stored at -20°C until isolation of RNA. Protein and Cr concentrations were measured using a Protein Assay Rapid Kit Wako and Laboassay Creatinine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

Quantitative Real-time PCR assay

RNA isolation from the pelleted urine and reverse transcription reaction were performed as described previously [8]. The absolute Pod-, Nep-, and AQP2-mRNA levels were quantified using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR master mix (Thermo Fisher Scientific Co. Ltd., Yokohama, Japan) and sample cDNA in a final volume of 15 μL per reaction. The following primers were used: podocin: forward 5'-AAGAGTAATTATATTCCGACTGGGACAT-3', reverse 5'-TGGTCACGATCTCATGAAAAGG-3'; nephrin: forward 5'-CAACTGGGAGAGACTGGGAGAA-3', reverse 5'-AATCTGACAACAAGACGGAGCA-3'; aquaporin-2: forward 5'-TGGGCCATATGTGCTATGGAGA-3', reverse 5'-AAGGACACTCAGGTGCCAGGA-3'. The thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C . All data were constructed from 0.5- μL samples analyzed in triplicate. The PCR product of each gene was used as a standard, and the standard curve was established with 10-fold serial dilution of the product. The transcript numbers were determined from linear regression of these standard curves. The detection limits of Pod- and Nep-mRNA expression were 100 copies/reaction. Therefore, we assumed that samples with undetectable levels of Pod- and Nep-mRNA, but a detectable level of AQP2-mRNA expression, contained 100 copies/reaction of the target gene for comparison of NPW vs. PW urine.

Data Expression Method

All assays were performed against standards to allow data to be expressed in terms of copies/ng of extracted RNA. As the urine sample volume varied from 3 to 130 mL, we first expressed data per milliliter of urine to compensate for urine concentration, and expressed data per milligram of urine Cr. All data are therefore expressed as copies per mg Cr.

Statistical analyses

Data are presented as the median (range). Statistical analyses were performed using the JMP10© statistical software package (SAS, Cary, NC). Mann–Whitney and Kruskal–Wallis tests were used for comparisons between two groups and three or more groups, respectively. Differences in frequencies were examined using Fisher’s exact test. The Spearman’s rank order correlation was used to test associations between two variables. In all analyses, $P < 0.05$ was taken to indicate statistical significance. However, a significant finding regarding a linear correlation between two variables was defined as that meeting both $P < 0.05$ and correlation coefficient (R-value) > 0.2 .

RESULTS

The protein:Cr ratio (mg/mg) was significantly greater in urine from PW than NPW (Table 2), as proteinuria increases up to a protein:Cr ratio of 0.26 even in healthy uncomplicated pregnancies [13, 14]. The number of cells (other than blood cells) per mg Cr was significantly greater for urine from PW than NPW (Table 2 and Fig. 1). These observations suggested that the number of cells excreted in the urine was greater in PW than NPW. The Kruskal–Wallis test suggested that cell density was increased in later compared to earlier stages of pregnancy (Fig. 1).

The levels of AQP2-, Pod-, and Nep-mRNA expression were examined in all 87 urine specimens from all 51 participants. In all of the samples, AQP2-mRNA expression was detectable, while Pod- and Nep-mRNA expression were detectable in significantly smaller numbers of urine specimens from NPW than PW women (Table 2). These observations suggested that the number of podocytes was smaller in the urine of NPW than PW. Therefore, we assumed that absolute expression levels of Pod- and Nep-mRNA were at the detection limit in specimens with undetectable levels of Pod-mRNA or Nep-mRNA in further analyses for comparison of the two groups.

Cell density in the urine was significantly positively correlated with expression of all three of Pod-mRNA, Nep-mRNA, and AQP2-mRNA (Fig. 2). These observations suggested that podocytes and cells expressing AQP2 contributed significantly to total cell counts in the urine samples.

As expected based on the results shown in Fig. 1 and Fig. 2, the expression levels of all three mRNAs corrected by Cr were significantly higher in urine from PW than NPW (Fig. 3). These observations suggested that the numbers of podocytes as well as cells expressing AQP2-mRNA were greater in the urine of PW than NPW.

Urine expression level of AQP2-mRNA was significantly positively strongly correlated with both expression of Pod-mRNA and Nep-mRNA, suggesting that the number of urine cells expressing AQP2-mRNA increased with increasing podocyturia (Fig. 4).

DISCUSSION

This study comparing urine from NPW vs. PW yielded the following four observations: 1) the number of urine cells other than blood cells was increased in pregnancy; 2) cell density was significantly positively correlated with Pod-, Nep-, and AQP2-mRNA expression; 3) cell density was significantly higher in urine from PW than NPW; and 4) all Pod-, Nep-, and AQP2-mRNA expression levels normalized relative to Cr were significantly greater in urine from PW than NPW. All of these findings suggested that both podocyturia and number of urine cells expressing AQP2-mRNA were increased in healthy women with uncomplicated pregnancies compared to healthy non-pregnant women.

Even in preeclamptic pregnancies with increased podocyturia, the numbers of urine podocytes (median [25th – 75th percentile]) stained with antibodies against podocyte-specific proteins, such as a podocin, were reported to be only 0.28 (0.11 – 0.77) and 0.77 (0.28 – 1.78) per mg Cr for the 2nd and 3rd trimesters, respectively [7], and 3.1 ± 2.2 (mean $\pm$ SD) per mg Cr in another report [15]. Detection of podocytes on microscopy is difficult in the urine of healthy pregnant women [5, 7, 16, 17]; podocytes were detected in none of nine women with uncomplicated pregnancies [16], and none of 10 normotensive pregnant women [17]. None of 23 healthy pregnant women in the 3rd trimester showed urine podocyte counts > 0.85 per mg Cr [5]. In addition, in a study using liquid chromatography coupled with tandem mass spectrometry for detection of urinary podocytes, podocin tryptic peptide levels in normotensive pregnant women were less than one-tenth of those in women with preeclampsia (0.4 ± 0.04 vs. 4.6 ± 2.3 fmol/mg Cr, respectively) [15]. Therefore, it was considered difficult to compare microscopically detectable podocyturia between healthy non-pregnant women and healthy women with uncomplicated pregnancies. However, based on the present results, failure to detect podocytes stained with antibodies against podocyte-specific proteins on microscopy was considered not to suggest the complete absence of podocytes in the urine.

Increased urine cell number is a well-known phenomenon in pregnant women. These may consist of cells originating from the kidney, ureter, urinary bladder, urethra, vagina, and vulva. Generally, qPCR detects mRNA from viable or relatively intact cells, but it may also detect mRNA from apoptotic cells [6]. Therefore, the levels of Pod- and Nep-mRNA expression in pelleted urine samples may have reflected the number of podocytes in urine, i.e., the degree of podocyturia, but not fragments of podocytes. In this study, the numbers of urine cells other than blood cells were significantly positively correlated with Pod- and Nep-mRNA expression levels suggesting that podocytes accounted for some fraction of total urine cell counts even in urine with lower Pod- and Nep-mRNA levels. In addition, cell density and Pod- and Nep-mRNA expression levels were significantly higher for urine from PW than NPW suggesting increased

podocyuria in pregnancy, although some urine podocytes may have been apoptotic. Consistent with our results, Kelder *et al.* [6] reported that urine Pod-mRNA expression level was significantly higher for healthy pregnant women than healthy non-pregnant women. Thus, although the absolute number of urine podocytes may have been small in NPW as well as PW as reported previously [5, 7, 16, 17], the present study demonstrated increased podocyuria even in uncomplicated pregnancy compared to healthy non-pregnant women.

AQP2-mRNA expression level was also significantly positively correlated with cell density and was significantly higher in urine from PW than NPW. This suggested that the number of urine cells expressing AQP2-mRNA was also increased in uncomplicated pregnancies compared to healthy non-pregnant women. As AQP2 is expressed in the principal cells of the kidney connecting tubule and collecting duct [10], we speculated that the majority of urine cells expressing AQP2-mRNA originated from the kidney connecting tubule and collecting duct. In experiments in animal models, insults to the kidney cause increased AQP2-mRNA expression in the urine [11]. In an animal model of acute kidney injury, AQP2 expression levels in the collecting duct were consistently and significantly reduced [18].

The blood volume increases by approximately 40% in pregnancy [19], and effective renal plasma flow and glomerular filtration rate begin to increase in the early stage of pregnancy [20]. Thus, physiological hyperfiltration occurs even in normotensive pregnancies [9]. The glomerular podocytes are vulnerable to a stress of hyperfiltration [3]. This may explain the accelerated detachment of podocytes and cells expressing AQP2-mRNA from the kidney seen in this study.

Podocytes are terminally differentiated cells [1] and their turnover rate is very low [21, 22]. Detachment of greater numbers of podocytes from the glomeruli causes a long-lasting decrease in number of podocytes in the kidneys [23]. Podocyte depletion in the kidney is associated with end-stage renal disease in animal models [11, 24]. Women with preeclampsia are likely to shed more glomerular podocytes compared to women with uncomplicated pregnancies [5 – 8], and women with a prior history of preeclampsia are at increased risk of future end-stage renal disease [25]. The present results suggest that women are likely to shed glomerular podocytes even in uncomplicated pregnancies, although it is unclear at present whether this contributes to clinically significant podocyte loss in the kidneys.

In conclusion, the levels of podocyte-specific protein mRNA and AQP2-mRNA expression were increased even in urine from uncomplicated pregnancies compared to that from healthy non-pregnant women. This strongly suggests that women are more likely to shed glomerular podocytes and kidney connecting tubule and collecting duct cells after becoming pregnant.

Acknowledgements

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Declaration of conflicting interests

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1 Cell density in urine samples from non-pregnant and pregnant women

Data on cell density were available for 11 of 11 (100%) and 73 of 76 (96%) urine samples from healthy non-pregnant women (NPW) and healthy pregnant women (PW) with uncomplicated pregnancies, respectively. Numbers in parentheses indicate number of urine samples.

Figure 2 Correlations of urine cell density with urine Pod-, Nep-, and AQP2-mRNA expression normalized relative to creatinine

Urine samples were similar to those in Fig. 1. These results suggested that urinary cell density increased with increasing podocyturia and cells expressing AQP2-mRNA.

Figure 3 Urine expression levels of Pod-, Nep-, and AQP2-mRNA normalized relative to creatinine

All 87 urine from NPW and PW were examined for Pod-, Nep-, and AQP2-mRNA expression levels. Urine expression levels of all of Pod-, Nep-, and AQP2-mRNA were significantly higher in PW than NPW.

Figure 4 Correlation of urine AQP2-mRNA expression with urine Pod- and Nep-mRNA expression levels

AQP2-mRNA expression was strongly correlated with both Pod- and Nep-mRNA expression levels ($R > 0.8$).

Table 1. Demographic characteristics of study subjects

	NPW	PW	<i>P</i> -value
Number of women	11	40	
Age (years)	32 (23 – 36)	34 (24 – 42)	0.0928
Body height (m)	1.57 (1.49 – 1.66)	1.60 (1.45 – 1.68)	0.3495
Body weight (kg)*	51 (43 – 74)	55 (40 – 97)	0.7034
Body mass index (kg/m ²)*	21.0 (17.0 – 33.3)	20.3 (17.0 – 37.9)	0.9414
Total no. of urine samples	11	76	
No. of urine samples/person	1 (1 – 1)	2 (1 – 3)	
Timing of urine sampling			
1 st trimester (GW 5 – 13)	NA	22	
2 nd trimester (GW 14 – 27)	NA	28	
3 rd trimester (GW 28 – 38)	NA	26	

Data are presented as the median (range). GW, gestational week; NPW, healthy non-pregnant women; PW, healthy pregnant women with uncomplicated pregnancies; NA, not applicable;

*, pre-pregnancy level.

Table 2 Difference in levels of various variables between two groups

	NPW	PW	<i>P</i> -value
No. of urine samples	11	76	
Creatinine (Cr, mg/dL)	129 (82.3 – 278)	114 (19.7 - 326)	0.1104
Protein concentration (mg/dL)	0.7 (0.25 – 6.6)	3.6 (0.25 – 18.5)	0.0172
Protein:Cr ratio (mg/mg)	0.009 (0.001 – 0.030)	0.039 (0.001 – 0.249)	0.0001
No of cells ($\times 10^4$ /mg·Cr)*	0.56 (0.11 – 6.1)	2.68 (0.359 – 20.7)†	0.0004
Undetectable mRNA expression			
Aquaporin (AQP-mRNA)	0 (0.0%)	0 (0.0%)	1.000
Podocin (Pod-mRNA)	3 (27.3%)	1 (1.3%)	0.0109
Nephrin (Nep-mRNA)	10 (90.9%)	6 (7.9%)	< 0.0001

Data are presented as median (range); NPW, healthy non-pregnant women; PW, healthy pregnant women with uncomplicated pregnancies; *, cells other than blood cells; †, cell counts were not available in three of the 76 urine samples. In 12 women with three longitudinal samples, protein:Cr ratios were 0.022 (0.003 – 0.067), 0.042 (0.009 – 0.091), and 0.076 (0.019 – 0.167) for the 1st, 2nd, and 3rd trimesters, respectively ($P = 0.0017$, Kruskal–Wallis test).

Fig. 1

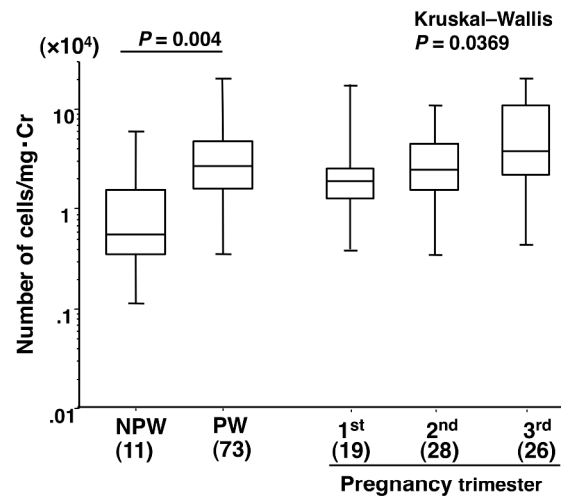


Figure 1 Cell density in urine samples from non-pregnant and pregnant women
Data on cell density were available for 11 of 11 (100%) and 73 of 76 (96%) urine samples from healthy non-pregnant women (NPW) and healthy pregnant women (PW) with uncomplicated pregnancies, respectively. Numbers in parentheses indicate number of urine samples.

297x209mm (300 x 300 DPI)

Fig. 2

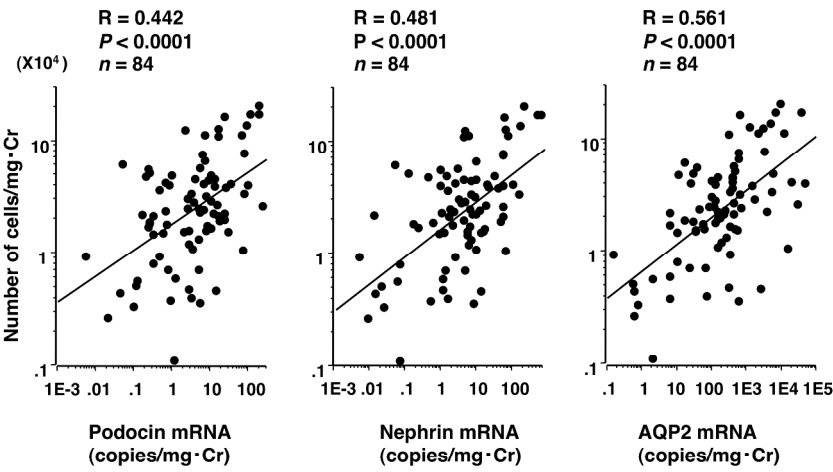


Figure 2 Correlations of urine cell density with urine Pod-, Nep-, and AQP2-mRNA expression normalized relative to creatinine
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Fig. 3

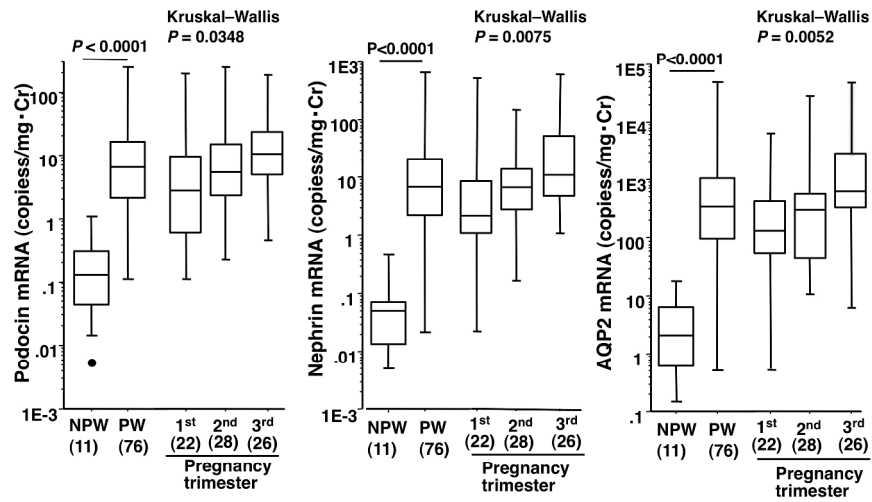


Figure 3 Urine expression levels of Pod-, Nep-, and AQP2-mRNA normalized relative to creatinine. All 87 urine from NPW and PW were examined for Pod-, Nep-, and AQP2-mRNA expression levels. Urine expression levels of all of Pod-, Nep-, and AQP2-mRNA were significantly higher in PW than NPW.

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Fig. 4

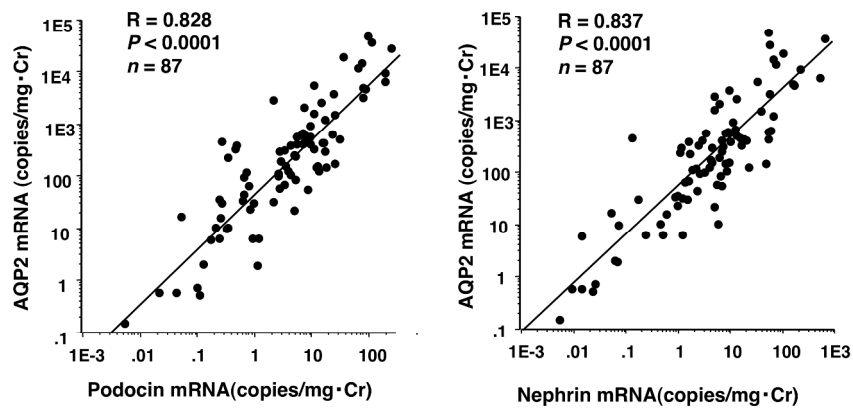


Figure 4 Correlation of urine AQP2-mRNA expression with urine Pod- and Nep-mRNA expression levels. AQP2-mRNA expression was strongly correlated with both Pod- and Nep-mRNA expression levels ($R > 0.8$).

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