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Title: Association between nephrinuria, podocyturia, and proteinuria in women with preeclampsia

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Running title: Pregnancy nephrinuria and podocyturia

Abstract

Aim: Podocyte depletion in the kidney is associated with end-stage kidney disease (ESKD). Preeclampsia (PE) increases the risk of ESKD in later life. This study was performed to determine whether nephrinuria (soluble nephrin in the urine) is correlated with proteinuria and or podocyturia (podocytes in the urine) in PE women.

Materials and Methods: Eighty-three urine samples, consisting of 45 and 38 samples from 27 normotensive and nine PE women, respectively, underwent simultaneous determination of nephrin, protein, and creatinine concentrations in the urine supernatant and quantitative analysis of podocyte-specific protein mRNA expression, including podocin (Pod-mRNA) and nephrin (Nep-mRNA), using RT-PCR in the pelleted urine. Nephrinuria and proteinuria were corrected by creatinine concentration. Pod- and Nep-mRNA expression levels were corrected by GAPDH.

Results: All of the nephrinuria, proteinuria, Pod-mRNA expression, and Nep-mRNA expression increased with advancing gestation in PE women, while not in normotensive women. The nephrinuria was strongly correlated with proteinuria ($R = 0.901$, $P < 0.001$), Pod-mRNA expression level ($R = 0.824$, $P < 0.001$), and Nep-mRNA expression level ($R = 0.724$, $P < 0.001$) in urine samples from PE women, while the nephrinuria was significantly correlated with proteinuria alone ($R = 0.419$, $P < 0.005$) in urine samples from normotensive women.

Conclusions: Nephrinuria reflected well degrees of proteinuria and podocyturia in PE women. This suggested that increased nephrinuria/proteinuria was associated with podocyte loss in the kidney of PE women.

Keywords: nephrin, podocyte injury, preeclampsia, proteinuric diseases, urine test

INTRODUCTION

Preeclampsia (PE), more than being proteinuric gestational hypertension alone, is a state of exaggerated systemic inflammation and remains a leading direct cause of maternal morbidity and mortality worldwide.¹ PE is a prominent risk factor for end-stage kidney disease (ESKD) in later life.² In the urine of women complicated with PE, both podocyuria (podocytes in the urine sediment) and nephrinuria (soluble nephrin in the urine) are detected.³⁻¹¹

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.^{12,13} Several diseases with proteinuria, including PE, show increased podocyuria.^{3-7, 14,15} Nephrin is a podocyte-specific transmembrane protein that is predominantly localized at the glomerular slit diaphragm of podocytes.¹⁶ Podocin is another podocyte-specific protein that contributes to the integrity of the filtration barrier. Thus, the degree of podocyuria can be monitored by quantification of podocin and nephrin mRNA expression in pelleted urine samples.^{3,7}

Nephrin expression is reduced in kidney biopsy specimens from PE women and in autopsy specimens from women that died from PE,^{17,18} and PE women show increased nephrinuria.⁸⁻¹¹ In humans and animal models, podocyte loss in the kidney is associated with reduced nephrin, but not podocin, mRNA expression in urine podocytes, suggesting that glomerular podocytes that have shed nephrin are likely to detach from the glomerular basement membrane and be excreted in the urine.^{7,19-21} Under such conditions, the shedding of nephrin from glomerular podocytes would lead to increased nephrinuria. If this is the case in PE pregnancy, nephrinuria would be correlated with podocyuria. However, there have been no previous studies regarding this issue.

The present study was performed to address the above issues using longitudinal urine samples provided by women who were asymptomatic at enrollment, but later developed PE.

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), a tertiary teaching hospital managing mainly high-risk pregnant women.

All 36 participants gave written informed consent prior to enrollment and fulfilled the

following two conditions: 1) neither significant proteinuria in pregnancy (**SPIP**) nor hypertension at enrollment with initial urine sampling; and 2) gave birth at Hokkaido University Hospital during the study period from May 2014 to October 2015. SPIP was defined as a protein:creatinine ratio (**P/Cr**, mg/mg) > 0.27 (corresponding to 30 mg/mmol) in spot urine specimens. Hypertension was diagnosed in women with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two occasions recorded more than 12 hours apart. PE was diagnosed in women that showed both hypertension and SPIP on and after gestational week (**GW**) 20.

All spot urine samples were coded and processed within 2 hours of collection. Urine samples were transferred to tubes and centrifuged at $700 \times g$ for 5 minutes. Urinary supernatant was stored at -20°C until measurement of protein, creatinine, and nephrin levels. The pelleted urine samples were suspended in *RNAlater* (Life Technologies, Carlsbad, CA, USA) and stored at -20°C until isolation of RNA. Protein and creatinine concentrations were measured using a Protein Assay Rapid Kit Wako and Laboassay Creatinine (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. Nephrin concentration was measured using an ELISA kit (Exocell Inc., Philadelphia, PA, USA). Urine samples were diluted in the range of 1:10 to 1:50 depending on proteinuria in the sample. The intra- and interassay coefficients of variation for nephrin were $< 10\%$. Protein and nephrin concentrations in the urine were corrected by urine creatinine concentration and were expressed as P/Cr (mg/mg) and nephrin:creatinine ratio (**N/Cr**, ng/mg).

Quantification of mRNA levels in the pelleted urine

RNA was isolated using the TRIzol method. The cell suspension in *RNAlater* was centrifuged at $20\,000 \times g$ for 3 minutes. Pellets were then dissolved with TRIzol (Life Technologies), and total RNA was extracted with RNeasy mini kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Following removal of contaminating genomic DNA with DNase, total RNA was purified using an RNeasy MinElute clean-up kit (Qiagen). Total RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The ratio of absorbance at 260/280 nm was used to assess RNA purity. Reverse transcription of total RNA was performed using a PrimeScript RT Reagent kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR) was performed to assess urinary expression of podocin mRNA (**Pod-mRNA**) and nephrin mRNA (**Nep-mRNA**). The housekeeping gene, GAPDH, was used to normalize the mRNA expression level of each target gene. The following oligonucleotide primer sequences were used: podocin, sense 5'-AAGAGTAATTATATTCCGACTGGGACAT-3' and antisense 5'-TGGTCACGATCTCATGAAAAGG-3'; nephrin, sense 5'-CAACTGGGAGAGACTGGGAGAA-3' and antisense 5'-AATCTGACAACAAGACGGAGCA-3'; and GAPDH, sense 5'-GAAGGTGAAGGTCGGAGTC-3' and antisense 5'-GAAGATGGTGATGGGATTTC-3'. Real-time PCR was performed using Power SYBR Green Master Mix (Invitrogen). The data were collected with an ABI Prism 7300

Sequence Detection System (Applied Biosystems, Foster City, CA, USA). 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. Relative quantification of target gene expression was performed using the $2^{-\Delta\Delta C_t}$ method. When a significant detectable increase in fluorescence did not occur in a specimen, the target gene was judged as undetectable in that specimen.

Statistical analyses

Data are presented as the median (range). Statistical analyses were performed using the JMP10© statistical software package (SAS, Cary, NC, USA). 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.

RESULTS

Nine of the 36 women developed PE; the nine women were enrolled in the study at GW 25.4 (19.0 – 36.1) at which time they were asymptomatic with regard to hypertension or SPIP, but developed SPIP at GW 32.0 (29.1 – 39.1) and hypertension at GW 32.3 (27.0 – 40.4) and gave birth at GW 33.4 (30.7 – 40.6) (Table 1). The remaining 27 were asymptomatic with neither SPIP nor hypertension throughout pregnancy. PE and normotensive women provided a total of 38 and 45 urine samples, respectively. Nineteen of the 38 urine samples from PE women were collected at latent phase with neither SPIP nor hypertension (Table 1). Of the 27 women with normotensive pregnancies, 14, 8, and 5 women provided one, two, and three urine samples, respectively.

All of the four measurements including N/Cr, P/Cr, Pod-mRNA expression level, and Nep-mRNA expression level increased with increasing GW in PE women (Fig. 1), while those did not in normotensive women. The N/Cr ratio (ng/mg) ranged from 8.75 to 17024.4 for PE women and 0.18 to 165.9 for normotensive women (Fig. 1). The P/Cr ratio (mg/mg) ranged from 0.004 to 6.549 for PE women and 0.001 to 0.153 for normotensive women. In urines from PE women compared to those from normotensive women, N/Cr and P/Cr levels were already significantly higher even before SPIP onset and all variable levels became significantly higher after PE onset (Fig. 2).

In the 38 urine samples from nine PE women, the N/Cr ratio increased linearly with increasing P/Cr ratio ($R = 0.901$, $P < 0.001$), Pod-mRNA expression level ($R = 0.824$, $P < 0.001$), and Nep-mRNA level ($R = 0.724$, $P < 0.001$) (Fig. 3, left). The P/Cr ratio also increased with increasing Pod-mRNA expression level ($R = 0.773$, $P < 0.001$) and Nep-mRNA level ($R = 0.784$, $P < 0.001$) (Fig. 3, right).

In the 45 urine samples from 27 normotensive women, the N/Cr ratio was significantly positively correlated with P/Cr ratio alone ($R = 0.419$, $P = 0.005$), but not with Pod-mRNA expression level or Nep-mRNA level (Fig. 4, left). The P/Cr ratio was not significantly correlated with Pod-mRNA expression level or Nep-mRNA level (Fig. 4, right).

DISCUSSION

The present study demonstrated for the first time that in PE pregnancy, the N/Cr ratio exhibited strong positive correlations (correlation coefficient [R-value] > 0.7) with P/Cr ratio, Pod-mRNA level, and Nep-mRNA level, suggesting that nephrinuria reflected well the degrees of proteinuria and podocyturia in PE women.

In this study, both podocyturia monitored by pelleted urine Pod-mRNA and Nep-mRNA levels and nephrinuria monitored by N/Cr ratio were increased in the urine of PE women with heavier proteinuria, confirming the results of previous studies. The increased podocyturia³⁻⁷ and nephrinuria⁸⁻¹¹ in PE pregnancy is an established phenomenon. However, to our knowledge, there have been no reports regarding an association between nephrinuria (urine nephrin concentration) and podocyturia (density of urine podocytes) in patients with podocytopathies, including diseases other than PE. As PE women exhibit a gradual increase in proteinuria,^{22,23} investigation of a longitudinal series of urine samples from PE women enabled analysis of this issue in this study.

The degree of nephrinuria reflected the degree of podocyturia monitored by urine sediment Pod-mRNA and Nep-mRNA expression levels in this study in PE women. As nephrin is a protein expressed specifically in the podocytes,¹⁶ soluble nephrin detected in the urine may have originated exclusively from podocytes. In an animal model with chronic kidney disease,¹⁹ nephrin expression was reduced in both glomerular and urine podocytes, and decreased nephrin expression in the urine podocytes was associated with pathological changes in the kidney histology with respect to glomerulosclerosis score, interstitial fibrosis score, and percentage podocyte depletion.²⁰ Reduced nephrin expression in the glomerular podocytes is also seen in the kidney of women that have died from PE.¹⁷ Reduced nephrin expression in the glomerular podocytes may be explained by shedding of nephrin from the glomerular podocytes and the glomerular podocytes with less nephrin expression is suggested to be likely to detach from the glomerular basement membrane.^{7,19-21} Thus, this study suggested that degree of nephrinuria can predict degree of kidney injury.

Soluble endoglin (sEng) is suggested to be responsible for podocyte detachment from the glomerular basement membrane.²⁴ The placenta-derived sEng level is increased in PE women²⁵ and suggested to be pathogenic for PE causing endothelial cell dysfunction, vascular hyperpermeability, and hypertension.^{24, 25} If this is the case, plasma sEng levels also may have been correlated with degree of both nephrinuria and podocyturia in PE participants in this study.

Nephrinuria as well as proteinuria were correlated well with podocyturia in this study. The clinical implications of this finding are that non-invasive urine test to determine nephrin/protein concentration may be useful to assess glomerular podocyte injury and podocyte depletion in the kidney. As podocytes are terminally differentiated cells²⁶ and their turnover rate is very low,^{27, 28} the detachment of podocytes from the glomeruli causes a long-lasting decrease in number of podocytes in the kidneys.²⁹ Experiments in

animal models suggested that podocyte depletion in the kidney is associated with ESKD irrespective of the cause of kidney disease.^{19, 20} Indeed, the kidneys of PE women are suggested to have decreased numbers of podocytes^{17, 18} and PE is a prominent risk factor for ESKD.² Aging is a risk factor for ESKD³⁰ and older humans exhibit greater podocyturia compared to younger subjects.²¹

Detailed simultaneous determinations of changes in urinary N/Cr, P/Cr, Pod-mRNA, and Nep-mRNA revealed that urinary N/Cr was strongly, significantly, and positively correlated with all of P/Cr, Pod-mRNA, and Nep-mRNA in PE pregnancies, but not in normotensive pregnancies. Degree of podocyturia monitored by Pod- and Nep-mRNA expression levels continued to increase during pregnancy of PE women in this study. These results suggested that increased nephrinuria/proteinuria was associated with podocyte loss (decreased number of podocytes) in the kidney of PE women.

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Declaration of conflicting interests

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1 Changes in N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression according to gestational week

Data of nine PE women (left panels) and 27 normotensive women (right panels) are plotted against gestational week. All PE women exhibited gradual increases in N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression during pregnancy.

Figure 2 N/Cr ratio, P/Cr ratio, Pod-mRNA, and Nep-mRNA expression levels in PE women before and after SPIP occurrence

Nineteen of the 38 urine samples from PE women were collected before SPIP onset.

Figure 3 Correlations between N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression in urines from PE women

The N/Cr ratio level was significantly positively correlated with all of the P/Cr ratio, Pod-mRNA level, and Nep-mRNA level in urines from PE women (left panels). The P/Cr ratio was also significantly positively correlated with both Pod-mRNA and Nep-mRNA levels (right panels). These results suggested that nephrinuria increased with increasing proteinuria and podocyturia, suggesting that nephrinuria reflected both proteinuria and podocyturia in PE pregnancies.

Figure 4 Correlations between N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression in urines from normotensive women

In urines from normotensive pregnancies, the N/Cr ratio level was significantly positively correlated with the P/Cr ratio level alone (left panels). However, the correlation between them was much weaker than that in PE pregnancies (R-values of 0.419 for the former vs. 0.901 for the latter [see [Fig. 3](#)]). The P/Cr ratio was not significantly correlated with Pod-mRNA level or Nep-mRNA level. These results suggested that the degree of nephrinuria did not reflect the degree of podocyturia in normotensive women without SPIP.

Table 1. Demographic characteristics of study subject

	Preeclampsia	Normotensive	<i>P</i> -value
Number of women	9	27	
Maternal age (years)	39 (19 – 43)	34 (26 – 42)	0.5686
Nulliparous	8 (89%)	16 (59.3%)	0.1025
Gestational week (GW)			
Enrolment	25.4 (19.0 – 36.1)	13.7 (10.6 – 38.9)	0.0777
SPIP onset	32.0 (29.1 – 39.1)	NA	
Hypertension onset	32.3 (27.0 – 40.4)	NA	
Delivery	33.4 (30.7 – 40.6)	38.3 (36.7 – 41.9)	< 0.001
< 37	6 (67%)	1 (3.7%)	< 0.001
Infant birthweight (kg)	2.06 (1.2s – 2.81)	2.93 (2.57 – 3.47)	< 0.001
Total no. of urine samples	38	45	
No. of urine samples/person	3 (3 – 9)	1 (1 – 3)	
Timing of urine sampling			
1 st trimester (GW 5 – 13)	0/[0]	14/[14]	
2 nd trimester (GW 14 – 27)	13/[7]	13/[13]	
3 rd trimester (GW 28 – 38)	25/[9]	18/[15]	
Latent phase	19/[9]	NA	

Data are presented as the median (range). Numbers in square brackets indicate the number of women that provided urine samples. SPIP, significant proteinuria in pregnancy defined as a protein-to-creatinine ratio (mg/mg) > 0.27. Latent phase was defined as clinical phase before the development both SPIP and hypertension.

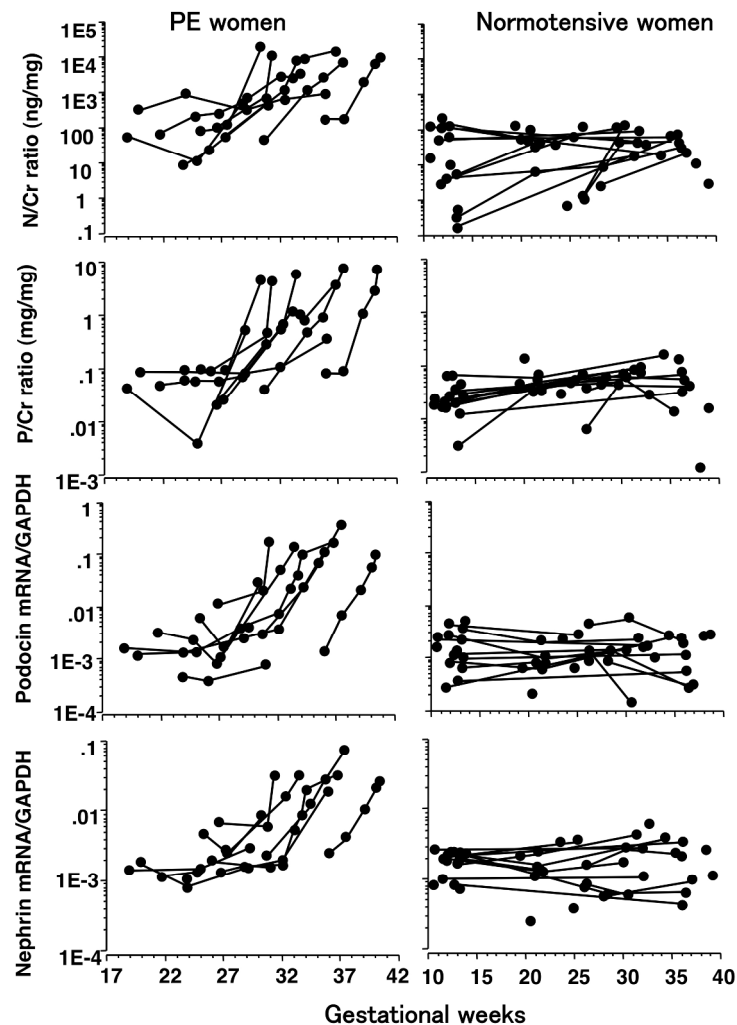


Figure 1 Changes in N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression according to gestational week

Data of nine PE women (left panels) and 27 normotensive women (right panels) are plotted against gestational week. All PE women exhibited gradual increases in N/Cr ratio, P/Cr ratio, Pod-mRNA expression, and Nep-mRNA expression during pregnancy.

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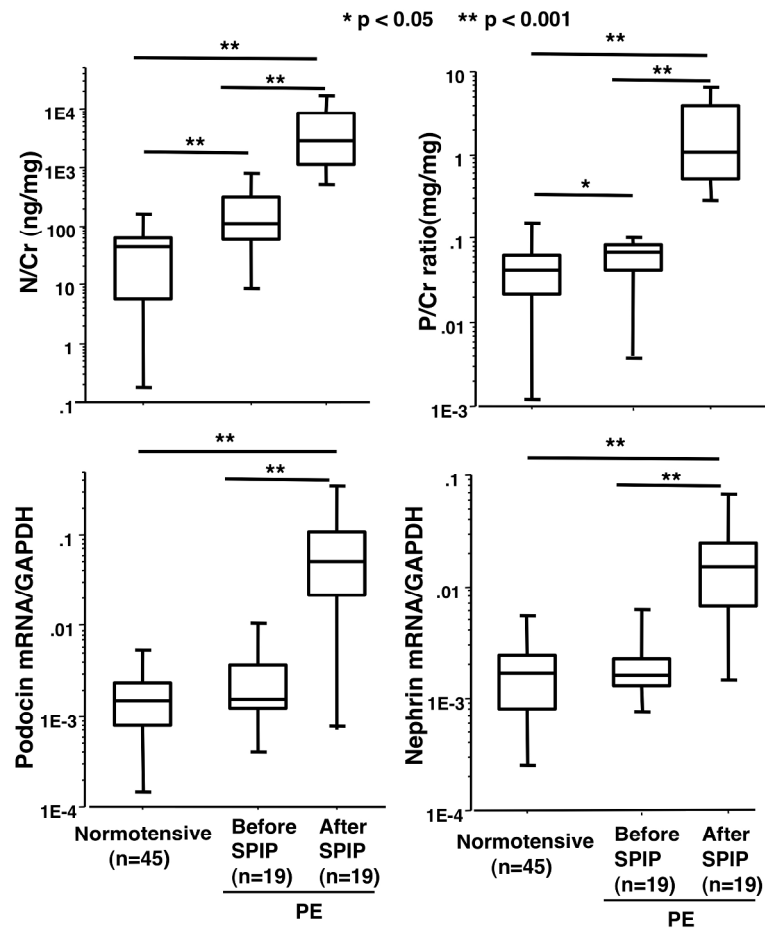


Figure 2 N/Cr ratio, P/Cr ratio, Pod-mRNA, and Nep-mRNA expression levels in PE women before and after SPIP occurrence
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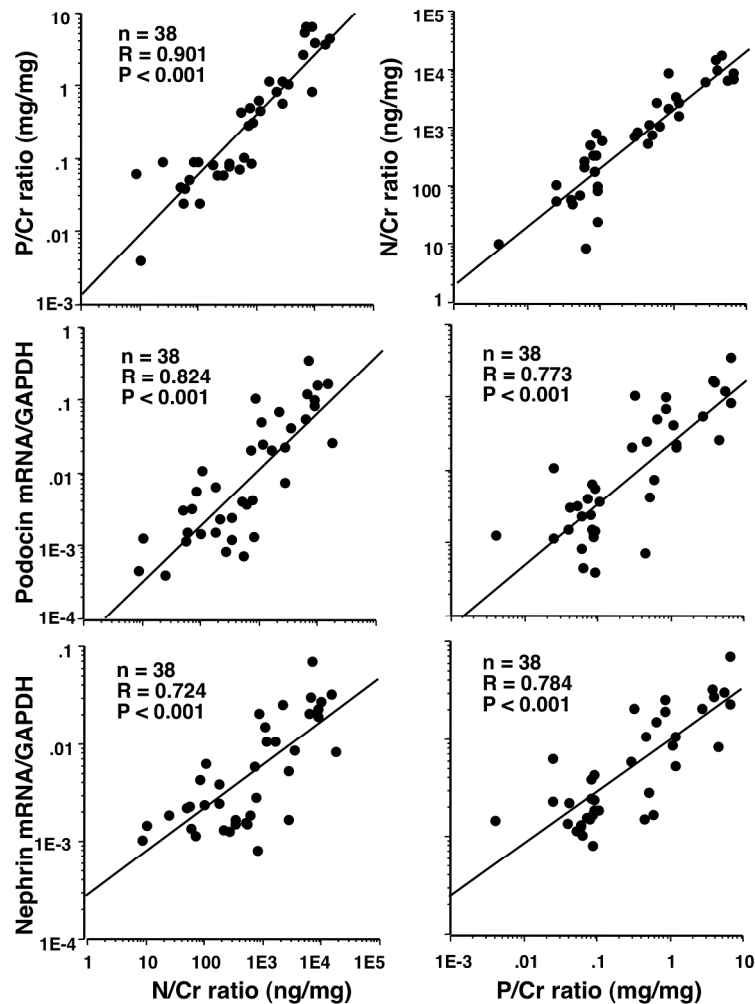


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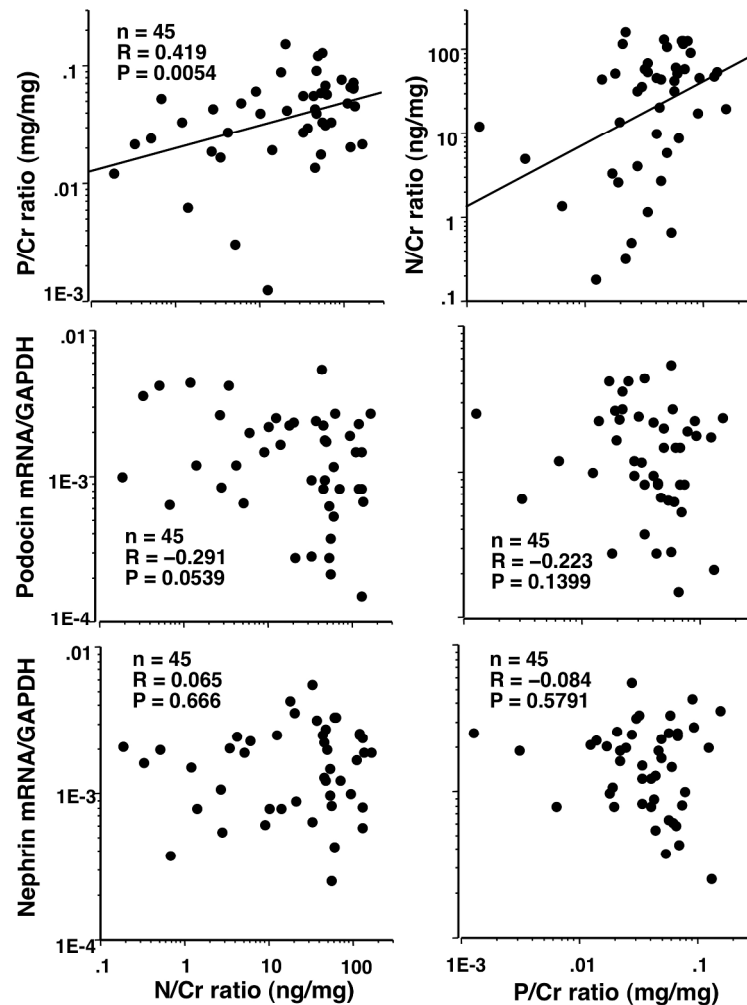


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