Postpartum podocyturia following preeclamptic pregnancy

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Running title: Postpartum podocyturia

Abstract

Aim: Urine podocin mRNA expression and urine podocin:nephrin mRNA expression ratio (PNR) increase with increasing proteinuria during pregnancy complicated with preeclampsia (PE). These observations suggested that urine podocytes with reduced nephrin mRNA expression are abundant in pathological podocyturia. This study was performed to determine postpartum changes in podocyturia and PNR in relation to proteinuria after preeclampsia (PE).

Methods: A total of 137 peripartum urine specimens, consisting of 72 and 65 from 24 and 30 women with PE and normotensive control pregnancies (NCP), respectively, were studied. Determination of urine protein and creatinine concentrations and quantitative analyses of podocyte-specific podocin and nephrin mRNA expression were performed using RT-PCR in pelleted urine samples. Podocyturia was monitored by urine podocin mRNA expression level. Podocyturia and proteinuria were normalized by urine creatinine concentration.

Results: Podocyturia and urine PNR decreased with decreasing proteinuria as well as increasing time after delivery in the urine from PE women. However, in comparison of postpartum urine with physiological proteinuria of protein:creatinine ratio (P/Cr, g/g) ranging from 0.005 to 0.1 collected from PE vs. NCP women, both podocyturia and PNR were significantly greater for the urine from PE than NCP women, although P/Cr was similar in both groups (median, 0.037 for PE vs. 0.029 for NCP).

Conclusions: Podocyturia decreases with decreasing proteinuria in PE women after childbirth. However, in PE women, pathological podocyturia consisting of podocytes with decreased nephrin mRNA expression persisted even after proteinuria decreased to a level comparable to that in NCP women.

Key words: podocin, nephrin, podocyte injury, pregnancy, proteinuric disease

INTRODUCTION

Podocytes that line the outer aspect of the glomerular basement membrane (GBM) form the final barrier to protein loss from the kidney.[1] Podocytes detach from the GBM and are present in the urine (as podocyturia) in various proteinuric diseases, including preeclampsia (PE).[2 – 7] As podocytes are terminally differentiated cells[1] and their turnover rate is very low,[8,9] the detachment of glomerular podocytes from the GBM contributes to a decrease in number of glomerular podocytes in the kidneys.[1,10]

Podocyturia can be monitored by mRNA expression levels of podocyte-specific proteins, such as podocin and nephrin,[7,11,12] and increases with increasing proteinuria in pregnancy complicated with PE.[7,11,12] However, certain stimuli cause alteration of the phenotype of glomerular podocytes, i.e., reduced nephrin mRNA expression in the glomerular podocytes, in animal models, and such glomerular podocytes with reduced nephrin mRNA expression are suggested to be likely to detach from the GBM resulting in increases in urine podocin:nephrin mRNA ratio (PNR).[13] Indeed, urine PNR increases with increasing podocyturia in PE women.[7] Thus, podocytes with reduced nephrin mRNA expression are abundant in pathological podocyturia in PE women. Therefore, urine podocin mRNA level, but not nephrin mRNA expression level, reflects the degree of podocyturia.

Proteinuria decreases postpartum and podocyturia is suggested to decrease postpartum in PE women.[14] However, postpartum changes in podocyturia and urine PNR in relation to proteinuria remain to be studied. The present study was performed to determine postpartum changes in podocyturia monitored by podocin mRNA expression level and urine PNR in PE women.

METHODS

Participants

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. All women gave written informed consent prior to participation in this study. A total of 54 women consisting of 30 with normotensive control pregnancies (NCP) and 24 with PE participated in this study and gave birth at Hokkaido University Hospital during the study period from June 2014 to July 2016 (Table 1). No women with known hypertension or renal diseases were included in this study. PE was diagnosed in women that showed both hypertension and significant proteinuria in pregnancy (SPIP) for the first time on or after gestational week (GW) 20. Test result was exclusively negative on dipstick for proteinuria performed at antenatal care before GW 20 in all 24 PE women. Protein:creatinine ratio (P/Cr, g/g) was determined before GW 20 in 10 of the 24 with later PE; median (range) P/Cr was 0.042 (0.002 – 0.064) for the 10 women at GW 17.9 (13.7 – 19.7). SPIP and hypertension was noted for the first time at GW 32.2 (24.4 – 39.1) and 32.6 (24.4 – 40.4), respectively in the 24 PE women (Table 1). Thus, no women with hypertension or SPIP before GW 20 were included in this study. SPIP was defined as P/Cr > 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. A total of 47 antepartum and 90 postpartum urine specimens were provided by the 54 women participating in the study (Table 1). Postpartum urine samples were collected on various number of days after childbirth. Postpartum day (PPD) 5 – 9, 20 – 45, and 50 – 120 were designated as "PP1," "PP2," and "PP3," respectively.

All 137 urine specimens were coded and processed within 2 hours of collection. Urine samples were centrifuged at 700 × *g* for 5 minutes. Urinary supernatant was stored at –20°C until measurement of protein and creatinine (Cr) levels. The pelleted urine samples were suspended in RNA*later* (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. Urine protein concentration was corrected by urine Cr and expressed as P/Cr (g/g).

Quantitative Real-time PCR assay

RNA isolation from the pelleted urine and reverse transcription reaction were performed as described previously.[7] The absolute podocin, nephrin, and aquaporin 2 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 dilutions of the product. The transcript numbers were determined from linear regression of these standard curves. As quantity of RNA extracted from the pelleted urine varied, aquaporin-2 mRNA was used as a kidney-specific reference gene unrelated to glomerular injury. The limit of detection for podocin, nephrin, and aquaporin-2 mRNA expression was 100 copies/reaction. In this study, only urine specimens with more than 500 copies of aquaporin-2 mRNA per real-time PCR were used for analyses. Samples with undetectable levels of podocin or nephrin mRNA, but a detectable level of aquaporin-2 mRNA expression, were assumed to contain 100 copies/reaction of the target.

Statistical analyses

Data are presented as the median (range). Statistical analyses were performed using the

JMP10© statistical software package (SAS, Cary, NC). The Kruskal–Wallis test with Bonferroni correction was used for comparisons between three or more groups. 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, significant findings regarding a linear correlation between two variables were defined as those meeting both $P < 0.05$ and correlation coefficient (R) > 0.2.

RESULTS

Aquaporin 2 mRNA expression was detectable with more than 500 copies per real-time PCR in all 137 urine specimens. In 24 ante- and 48 postpartum urine specimens from PE women, podocin mRNA expression was undetectable in none (0.0%) and one (2.1%), respectively, and nephrin mRNA was undetectable in none (0.0%) and one (2.1%), respectively. In 23 ante- and 42 postpartum urine specimens from NCP women, podocin mRNA expression was undetectable in two (8.7%) and five (11.9%), respectively and nephrin mRNA was undetectable in one (4.3%) and six (14.3%), respectively.

Changes in P/Cr, podocin and nephrin mRNA expression levels, and PNR in PE and NCP women according to number of days after childbirth

The podocin and nephrin mRNA expression levels decreased with increasing number of days after childbirth in PE women (Fig. 1). This indicated that number of podocytes monitored by podocin mRNA expression decreased with increasing number of days after childbirth in PE women. The PNR was markedly high antepartum and at PP1 (Fig. 1). This indicated that in each podocyte in the urine with abundant podocytes, nephrin mRNA expression was markedly reduced compared to podocin mRNA expression. However, PNR decreased with decreasing number of urine podocytes. This indicated that gradual increase in nephrin mRNA expression occurred in each urine podocyte with increasing number of days after childbirth in PE women.

In NCP women, all variable levels of the 23 antepartum urines except nephrin mRNA expression decreased significantly in the 42 postpartum urines; P/Cr of 0.05 (0.004 - 0.109) decreased to 0.02 (0.001 - 0.105) ($P = 0.0022$), podocin mRNA expression of 4.45 (0.14 - 51.2) decreased to 0.63 (0.056 - 71.5) ($P = 0.0005$), and PNR of 1.65 (0.17 - 11.3) decreased to 0.69 (0.05 - 2.70) ($P < 0.0001$). Nephrin mRNA expression levels were 2.87 (0.12 - 31.7) and 1.46 (0.06 - 26.8) ($P = 0.4104$) for antepartum and postpartum urines, respectively. Postpartum decease in podocin mRNA expression in NCP women suggested an increased podocyturia even in NCP pregnancies, confirming results of our previous study.[15]

Correlation of P/Cr with podocin mRNA expression level, nephrin mRNA expression level, and PNR in postpartum urine samples from NCP and PE women

All of podocin mRNA expression level, nephrin mRNA expression level, and PNR decreased with decreasing P/Cr in postpartum urine samples from PE women (Fig. 2).

This indicated that postpartum podocyturia monitored by podocin mRNA expression decreased with decreasing proteinuria. However, the slope of the linear regression line was more gentle for PNR than for podocin mRNA. This suggested that nephrin mRNA expression level was still reduced in each urine podoctye even after proteinuria decreased to a level comparable to that in NCP women.

Comparison of podocin and nephrin mRNA expression levels, and PNR in postpartum urine samples with physiological proteinuria from NCP vs. PE women

To determine whether degree of podocyturia and podocyte phenotype in the urine from PE women differed from those in the urine from NCP women, urine samples with P/Cr ranging from 0.005 to 0.1 were chosen from both groups to match P/Cr level (see vertical bars in Fig. 2). In 31 vs. 19 urine samples with P/Cr of 0.005 – 0.1 from NCP vs. PE women, respectively, P/Cr was similar, but all of podocin mRNA expression level, nephrin mRNA expression level, and PNR were significantly greater in the urine samples from PE than NCP women (Fig. 3). As suggested based on Fig. 2, high levels of podocin mRNA expression, nephrin mRNA expression, and PNR were characteristic features in SPIP urine samples (Fig. 3). Thus, even in the urines of PE women containing physiological proteinuria, podocyturia was still high and nephrin mRNA expression in each urine podocyte was still low compared to urines of NCP women.

DISCUSSION

This study was performed to examine postpartum urine samples, and the findings can be summarized as follows: (1) podocyturia monitored by podocin mRNA expression level as well as nephrin mRNA level decreased with decreasing proteinuria as well as increasing number of days after childbirth in women following PE pregnancy; (2) PNR decreased with decreasing proteinuria as well as increasing number of days after childbirth in women following PE pregnancy; and (3) both podocyturia and PNR were greater in the urine from women following PE pregnancy than in those from women following NCP, even though P/Cr was similar.

Generally, changes occurring in pregnant women, e.g., maternal weight, the circulating blood volume, and blood pressure in PE women, gradually return to non-pregnant level after childbirth. As podocyturia increases with increasing proteinuria until childbirth in PE women,[7] it was expected that podocyturia would decrease with decreasing proteinuria after childbirth, and this was verified in the present study.

PNR also increases with increasing proteinuria until childbirth in PE women.[7] This was interpreted as indicating that as yet unknown factor(s) associated with PE caused a phenotypic alteration of glomerular podocytes, i.e., reduced nephrin mRNA expression, and glomerular podocytes with reduced nephrin mRNA expression were likely to detach from the GBM, resulting in increased podocyturia in which podocytes with reduced podocin mRNA were abundant.[7] Thus, abundant podocytes with reduced nephrin mRNA expression were characteristic of pathological podocyturia associated with PE. This process occurring during pregnancy was also ameliorated by childbirth; the PNR decreased gradually with increasing number of days after childbirth as well as

decreasing proteinuria in this study. These observations suggested that phenotype alteration of glomerular podocytes (i.e., decease in nephrin mRNA expression in glomerular podocytes) gradually diminished with increasing number of days after childbirth in women following PE.

However, podocyturia and PNR were still significantly greater even after proteinuria decreased to a level comparable to that in women following NCP in this study. The 19 postpartum urine samples with P/Cr ranging from 0.005 to 0.1 were sampled approximately 1 month postpartum (see legend for Fig. 3). This suggested that pathological processes leading to decreased nephrin mRNA expression in the glomerular podocytes were still active even after normalization of proteinuria in women following PE pregnancy. Thus, pathological podocyturia persisted for approximately 1 month postpartum in women following PE pregnancy in this study, consistent with findings by White *et al*[14] in which frequency of podocytuira at 5 to 8 weeks postpartum was significantly greater for PE women than for NCP women.[14] These results suggested that not only PE pregnancy but also a certain postpartum period following PE may have contributed to the decrease in number of glomerular podocytes. As podocytes are terminally differentiated cells[1] and their turnover rate is very low,[8,9] the detachment of podocytes from the GBM causes a long-lasting decrease in number of podocytes in the kidneys.[1,10] Experiments in animal models suggested that podocyte depletion in the kidney is associated with end-stage kidney disease (ESKD) regardless of the cause of kidney disease.[13,16] Indeed, decreased nephrin expression is seen in the kidneys of PE women[17,18] suggesting decreased number of podocytes in the kidney and or decreased nephrin expression in the glomerular podocytes of PE women. PE is a prominent risk factor for ESKD[19]; among women with experience of three or more pregnancies, those with one PE pregnancy have a relative ESKD risk of 6.3 (95% CI, 4.1 – 9.9), while those with two PE pregnancies have a relative ESKD risk of 15.5 (95% CI, 7.8 – 30.8) compared to women with no PE pregnancies.[19]

In an animal model, angiotensin receptor blocker (ARB) showed a renoprotective effect via podocyte protection,[20] and was indeed shown to confer significant renal benefits in patients with type 2 diabetes and nephropathy reducing proteinuria.[21] Although contraindicated in pregnancy,[22] use of ARB can be considered for renoprotection in postpartum women after PE pregnancy.

In conclusion, this study demonstrated that podocyturia decreased with decreasing proteinuria in women following PE. However, this study also demonstrated that in women following PE, pathological podocyturia consisting of abundant podocytes with decreased nephrin mRNA expression persisted even after proteinuria decreased to a level comparable to that in women following NCP. This suggested long-lasting podocyte injury in the kidney after PE pregnancy.

Acknowledgments

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Declaration of conflicting interests

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Postpartum changes in urine variables in PE women

*, $P < 0.05$ between two values with the same symbol. Shadow areas indicate levels below median value for the 23 ante- and 42 postpartum urines from NCP women. Urine

samples were classified into four categories according to timing at collection; antepartum (AP) and postpartum day 5 – 9 (PP1), 20 – 45 (PP2), and 50 – 120 (PP3). The numbers of women with longitudinal paired urine samples available are indicated. For example, 16 women provided urine samples collected at AP and PP1. Proteinuria monitored by P/Cr, podocyturia monitored by podocin as well as nephrin mRNA expression level, and podocin:nephrin mRNA expression ratio (PNR) decreased with increasing number of days after childbirth.

Figure 2. Correlation of P/Cr with podocin and nephrin mRNA expression levels and PNR in postpartum urine samples

The red oblique line indicates the linear regression line between values of x and y axes for urine samples from PE women. Postpartum P/Cr was significantly positively correlated with urine podocin mRNA expression level, nephrin mRNA expression level, and PNR in urine samples from PE women, but not in urine samples from NCP women. Thus, neither podocyturia nor PNR changed significantly according to P/Cr in the 42 postpartum urine samples from NCP women. Three vertical bars indicate P/Cr levels of 0.005, 0.1, and 0.27. Urine samples with P/Cr ranging from 0.005 to 0.1 and those with SPIP (P/Cr > 0.27) were used for further analyses as shown in Fig. 3.

Figure 3. Comparison of urine samples with physiological proteinuria between those from NCP vs. PE women

Of 90 postpartum urine samples (48 and 42 urine samples from PE and NCP women, respectively), 21 urine samples sampled on PPD 7 (5 – 94) from PE women exhibited SPIP and 50 urine samples (consisting of 31 urine samples sampled on PPD 26 (5 – 105) from NCP women and 19 urine samples sampled on PPD 35 (26 – 117) from PE women) exhibited physiological proteinuria with P/Cr ranging from 0.005 to 0.1 (see Fig. 2). High podocyturia monitored by podocin mRNA as well as nephrin mRNA expression and high PNR were characteristic features of SPIP urine samples; median (range) P/Cr was 0.61 (0.29 – 8.42), median podocin mRNA expression level was 64.7 (0.49 – 20276), and nephrin mRNA expression level of 40.9 (0.30 – 5823), and PNR was 2.7 (0.09 – 10.1) for the 21 SPIP urine samples. Among urine samples with P/Cr ranging from 0.005 to 0.1 from NCP vs. PE women, median (range) podocin mRNA expression level (0.73 [0.06 – 8.57] vs. 4.23 [0.54 – 105]), median nephrin mRNA expression level (1.42 [0.06 – 15.8] vs. 2.85 [0.52 – 41.5]), and PNR (0.69 [0.05 – 2.69] vs. 2.16 [0.07 – 9.97]) were significantly lower for the 31 urine samples from NCP women than for the 19 urine samples from PE women, although median P/Cr was similar (0.029 [0.001 – 0.098] vs. 0.037 [0.054 – 0.092], respectively).

Table 1. Demographic characteristics of 54 women

	Preeclampsia (PE)	Normotensive control pregnancy (NCP)
No. of women	24	30
Maternal age (years)	37.0 (19 – 44)	31.5 (21 – 40)*
≥ 35	14 (58.3%)	9 (30.0%)
Nulliparous	22 (91.7%)	15 (50.0%)*
GW at onset of SPIP	32.2 (24.4 – 39.1)	NA
GW at onset of hypertension	32.6 (24.4 – 40.4)	NA
GW at delivery	35.2 (26.4 – 40.6)	38.0 (24.3 – 41.3)*
< 37	14 (58.3%)	2 (6.6%)*
Infant birthweight (g)	2039 (658 – 2860)	2908 (638 – 4225)*
No. of urine samples	72	65
Per woman	3 (2 – 4)	2 (1 – 3)*
Antepartum urine†	24	23
Postpartum urine	48	42
PP1 (PPD, 5 – 9)	16 (33.3%)	19 (45.2%)
PP2 (PPD, 20 – 45)	23 (47.9%)	16 (38.1%)
PP3 (PPD, 50 – 120)	9 (18.8%)	7 (16.7%)

Data are presented as the median (range). *, $P < 0.05$ vs. preeclampsia group; †, collected within 2 weeks before delivery; GW, gestational week; NA, not applicable; SPIP, significant proteinuria in pregnancy defined as protein:creatinine ratio (g/g) > 0.27. Postpartum day (PPD) 5 - 9, 20 - 45, and 50 - 120 were designated as PP1, PP2, and PP3, respectively.

Fig. 1

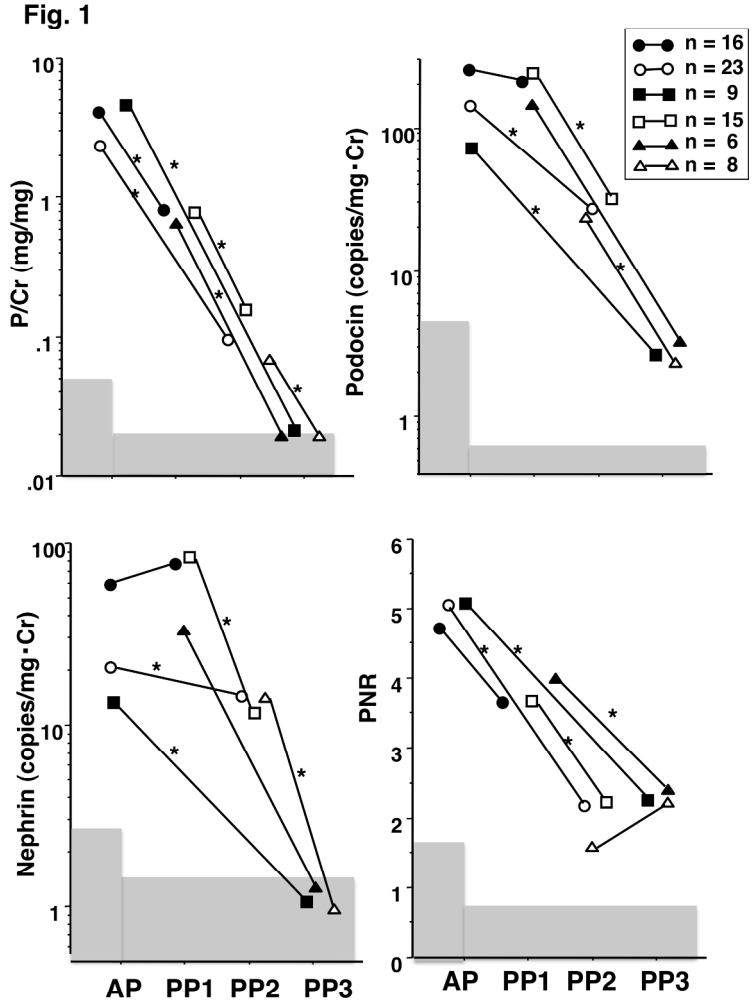

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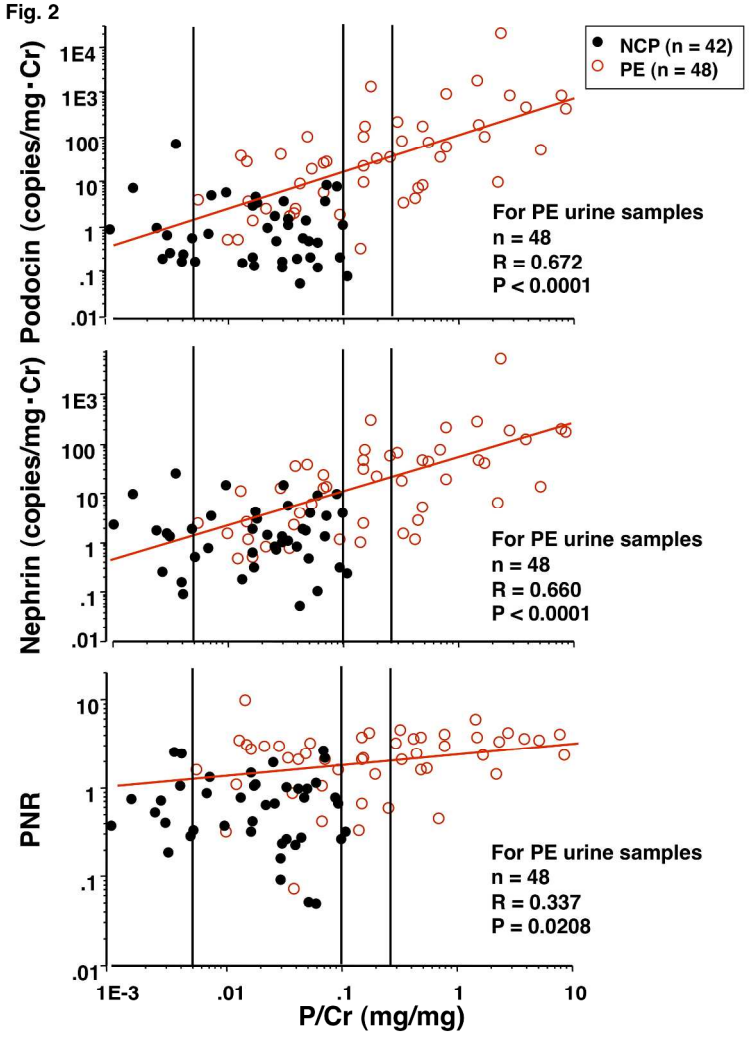
Fig. 2



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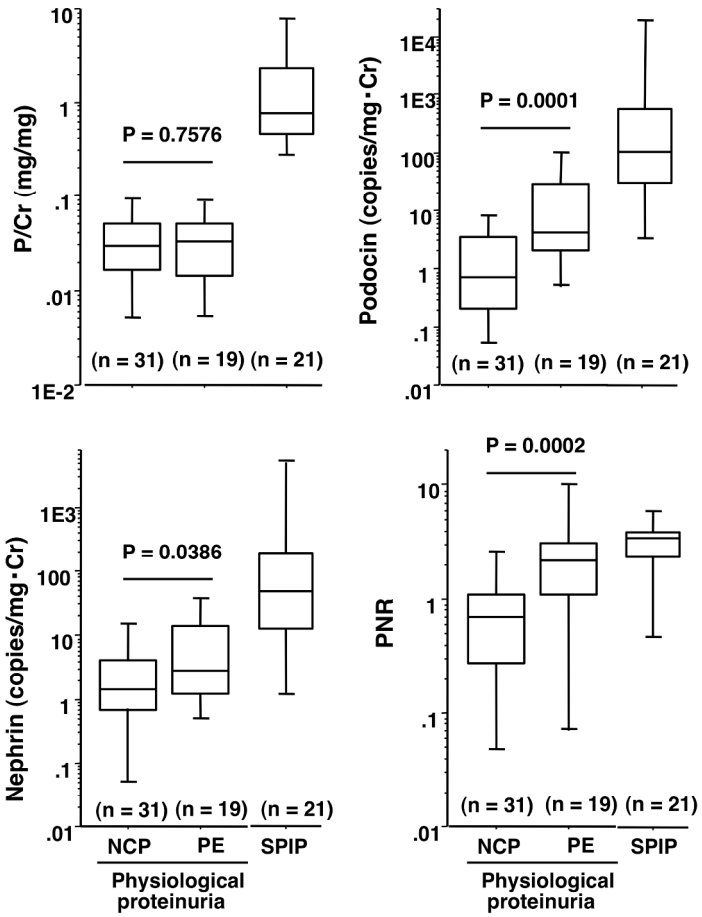

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