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의학박사 학위논문

**Proteomic identification of early urinary-biomarkers of acute  
kidney injury in preterm infants**

단백체학을 이용한 미숙아의 급성 신손상의 조기 소변

생체지표의 개발

2020 년 10 월

서울대학교 대학원

의학과 소아과학전공

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단백체학을 이용한 미숙아의 급성 신손상의 조기 소변 생체지표의  
개발

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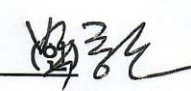
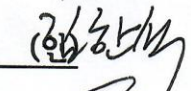
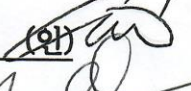
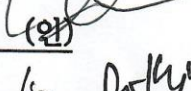
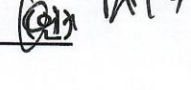

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**Proteomic identification of early urinary-biomarkers  
of acute kidney injury in preterm infants**

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## Abstract

# Proteomic identification of early urinary-biomarkers of acute kidney injury in preterm infants

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**Background:** The immature preterm kidney is likely to be vulnerable to acute kidney injury (AKI). However, the biomarkers currently used for AKI are not sensitive or specific and are also inadequate for the timely detection of AKI in preterm infants.

**Objective:** The objectives of this study were to identify novel urinary biomarkers of AKI using proteomic techniques, and to verify and validate that the candidates can serve as early predictive biomarkers for AKI.

**Materials:** The biomarker development process was divided the following 3 phases: Five paired urine samples were analyzed in the discovery phase. For verification, a prospective nested case-control study compared serial urinary proteomes of 16 patients with AKI and 16 patients without AKI. For validation, enzyme-linked immunosorbent assays (ELISAs) were performed using samples of the first-day urine from 14 preterm infants with AKI and from 14 matched control patients.

**Results:** In total, 1,810 proteins were identified in the discovery phase. Among those proteins, 174 were selected as the 1<sup>st</sup> targeted proteins. A total of 168 proteins were

quantified, and the levels of 6 were significantly increased in the AKI group in the verification phase. Using a clinical assay, the results were confirmed and validated using samples of the first-day urine after birth from the biorepository. Finally, ELISAs revealed that the levels of annexin A5, neutrophil gelatinase-associated lipocalin (NGAL), and protein S100-P were significantly higher in the samples of the first-day urine from patients with AKI than in those from patients without AKI.

**Conclusion:** Urinary annexin A5, NGAL and protein S100-P levels are promising biomarkers for early, accurate prediction of AKI in preterm infants.

**Keywords:** acute kidney injury, preterm infants, proteomics, biomarker

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## Introduction

Although data from neonatal acute kidney injury (AKI) research has been sparse until recently, previous epidemiological studies have suggested that AKI is common in neonates, and those with AKI are at risk for death and long-term chronic kidney disease<sup>1,2</sup>. Several single-center studies showed that the incidence of AKI in very-low-birth-weight infants was 15% to 40%, and the mortality was significantly higher in preterm infants with AKI than in those without AKI<sup>3-5</sup>. However, the incidence may be underestimated because of poor detection of nonoliguric renal failure in preterm infants and the serum creatinine (SCr)-based definition of AKI.

AKI is usually diagnosed indirectly by a measured increase in the level of SCr, which is thought to indicate a reduction in the glomerular filtration rate (GFR). However, creatinine is an unreliable indicator during acute changes in kidney function because changes in the SCr levels are a late consequence of kidney damage. SCr concentrations can vary widely based on age, sex, muscle mass, muscle metabolism, and medications<sup>6</sup>. In neonates, several endogenous and exogenous factors could contribute to the delay in diagnosis of AKI based on SCr<sup>7</sup>. Neonatal SCr levels during the first few days of life may not reflect their own kidney function but rather the mother's kidney function via the placenta. In addition, normal ranges of SCr change over time depending on gestational age and postnatal age<sup>8,9</sup>. SCr concentrations usually fluctuate significantly over the first weeks of a newborn's life<sup>10</sup>. In preterm infants in particular, there is a very wide distribution of normal SCr values that change over time, depending on the degree of prematurity<sup>8</sup>. In addition, measuring SCr frequently in small infants is not advised because of a concern about the amount of blood loss due to multiple blood samplings. Regardless of

these limitations, SCr still remains the standard tool used for the diagnosis of AKI because of the lack of appropriate biomarkers for AKI in neonates.

Recently, the application of innovative technologies, such as functional genomics and proteomics, has revealed several novel genes and gene products that are emerging as biomarkers. In contrast to the genome, which is unique and relatively stable, proteomes are cell- and tissue-specific and change over time in direct response to different situations. Typically, tissue analysis involves genomic approaches, whereas body fluids are best analyzed by proteomic techniques. Therefore, we considered that proteomics is a promising tool with which to search for biomarkers that directly reflect the pathological status in biological fluids<sup>11</sup>. Among the biological fluids, urine is most likely to contain biomarkers arising from the kidney. Urine is easily accessible in a large quantity without the use of invasive procedures and reflects the pathophysiological changes in the genitourinary tract and kidneys. Urinary proteins have been shown to remain stable long enough to enable performance of reliable proteomic analysis<sup>12</sup>.

The objectives of this study were 1) to identify novel urinary biomarkers of AKI using proteomic techniques, and 2) to verify and validate that the discovered candidates are early predictive biomarkers for AKI in extremely preterm infants.

## **Materials and methods**

### **Study design**

The biomarker development process was divided into the following 3 phases: the discovery, verification, and validation phases.

#### **1. Discovery phase**

We conducted a prospective cohort study at Seoul National University Children's Hospital between 2015 and 2017 that was designed to enroll preterm infants with birth weights <1,500 g or gestational ages <32 weeks who were treated with ibuprofen for patent ductus arteriosus. We collected serial urine samples during the 6 hours before starting ibuprofen treatment and after ibuprofen treatment. The SCr concentration was measured as part of routine preterm care. These data were used to select patients who developed AKI after ibuprofen treatment. To identify protein biomarker candidates in the discovery cohort, we performed label-free mass spectrometry (MS)-based protein quantification with 10 urine samples.

#### **2. Verification phase**

The biospecimen archive has been maintained by the neonatal intensive care unit at Seoul National University Children's Hospital since 2008. All preterm infants with birth weights <1,500 g or gestational ages <32 weeks who were admitted to Seoul National University Children's Hospital were eligible. All samples were prospectively collected from the time of birth until 36 weeks postmenstrual age or hospital discharge according to the established standardized procedures for sample collection.

We conducted a nested case-control study using urine samples from the biospecimen repository

to verify the identified candidates as biomarkers for predicting AKI. In this study, patients who developed AKI in the defined cohort were identified. Then, controls matched by gestational age and postnatal age were selected from the same cohort. To verify the candidate biomarkers identified in the discovery cohort, we implemented a targeted proteomics approach. In total, 36 samples were analyzed according to the data-independent acquisition (DIA) method.

### **3. Validation phase**

Using the biospecimen repository, we conducted another nested case-control study to validate the identified candidate biomarkers. To determine whether the candidate biomarkers can be used to detect prenatal AKI in preterm infants, we selected patients who developed AKI within 7 days after birth without any postnatal insults, such as administration of nephrotoxic medications, sepsis and hypotension. Then, for the control group, we selected those who did not develop AKI within 7 days after birth and matched them according to gestational age.

To evaluate the ability of the candidate biomarkers to predict AKI, enzyme-linked immunosorbent assays (ELISAs) were performed using the first-day urine samples after birth. Urinary biomarkers were measured in duplicate using commercially available ELISA kits. Urinary neutrophil gelatinase-associated lipocalin (NGAL), and galectin-3 were assessed by ELISA (Quantikine, R & D systems, Minneapolis, MN), with average confidences of variability (CVs) of 2.9% and 6.4%, respectively. Protein S100-P, annexin A5, cartilage intermediate layer protein 2 (CILP-2), and 6-phosphogluconolactonase (6-PGLS) were measured by ELISA kits (MyBiosource, San Diego, CA, USA), with average CVs of 12.9%, 8.4%, 5.5%, and 6.2%, respectively.

#### **AKI definition**

AKI was defined as an acute increase in the SCr of at least 0.3 mg/dL within 48 hours (stage 1 according to the AKI Network definition) or a persistent increase in SCr to  $\geq 1.7$  for 3 days after birth<sup>7,13</sup>. The AKI Network definition utilizes changes in serum creatinine concentration and urine output to characterize three levels of renal dysfunction. An abrupt (within 48 hours) reduction in kidney function defined as an absolute increase in serum creatinine concentration by either  $> 0.3$  mg/dL or an increase of  $\geq 50\%$  (1.5 fold from baseline) or reduction in urine output (documented oliguria of  $< 0.5$  mL/kg/hour for  $> 6$  hours). We did not use urine output criteria because premature infants often have nonoliguric renal failure due to immature tubular development. The controls were infants with at least 2 blood samples to confirm negative AKI status at the time of urine sample collection.

### **Urine collection and sample preparation**

Urine was collected by placing cotton balls at the perineum. The urine was extracted, centrifuged for 10 minutes to remove any cotton fibers and cellular elements, and then frozen at  $-70$  °C until sample evaluation. The starting volumes of the infant urine samples ranged from 200 to 500  $\mu$ L. After centrifugation at 3000 x g for 15 minutes at 4 °C, the urine supernatants were concentrated with an Amicon Ultra centrifugal filter device (3 kDa MWCO, Millipore) at 14,000 x g to a volume of  $\sim 50$   $\mu$ L. The protein content of the final concentrated solution was determined with the Bradford method (Bio-Rad Protein Assay, Bio-Rad). For the label-free quantification in the discovery phase, 50  $\mu$ L of urinary proteins were precipitated by adding a 6-fold volume of ice-cold acetone prior to the digestion step. For the DIA analysis in the verification phase, 25  $\mu$ L of urinary proteins per individual sample was used. The precipitated proteins were dissolved in sodium dodecyl sulfate-containing denaturation buffer.

After being heated at 95 °C, the denatured proteins were digested by a filter-aided sample preparation method<sup>14,15</sup>. The proteins were digested with trypsin (enzyme-to-protein ratio [w/w] of 1:100) at 37°C overnight. All samples were desalted using homemade C18-StageTips as previously described<sup>14,16</sup>. Then, a StageTip-based high-pH peptide fractionation was performed for the library samples in the discovery and verification phases. The desalted peptide samples and fractionated peptide samples were dried in a vacuum centrifuge and stored at -80 °C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

### **LC-MS/MS analysis**

All LC-MS/MS analyses, which were performed by the data-dependent acquisition (DDA) and DIA methods, were conducted with an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA, USA) coupled to a Q-Exactive Plus mass spectrometer (Thermo) as previously described with some modifications. Peptide samples were separated on a two-column system with a trap column and an analytical column (75 µm X 50 cm) with 120-minute gradients from 7% to 32% acetonitrile at 300 nl/minutes. The column temperature was maintained at 60°C using a column heater. The column eluent was delivered to the Q-Exactive Plus via nanoelectrospray. In the DDA method for label-free quantification, a survey scan (350 to 1650 m/z) was acquired with a resolution of 70,000 at m/z 200. A top-20 method was used to select the precursor ion with an isolation window of 1.2 m/z. The MS/MS spectrum was acquired at an HCD-normalized collision energy of 30 with a resolution of 17,500 at m/z 200. The maximum ion injection times for the full and MS/MS scans were 20 and 100 ms, respectively. The hyper reaction monitoring (HRM) DIA method consisted of a survey scan at 35,000 resolution from 400 to 1,220 m/z (automatic gain control target of  $3 \times 10^6$  or 60-ms injection time). Then, 19 DIA windows were acquired at a resolution of 35,000 with an automatic gain control target of  $3 \times 10^6$  and auto

injection time<sup>17</sup>. The stepped collision energy was 10% at 27%.

### **Data processing for label-free quantification**

All MS raw files were processed by using the interface of MaxQuant (version 1.5.3.1)<sup>18</sup>. MS/MS spectra from the Human UniProt protein sequence data set (December 2014, 88,657 entries) were searched using the Andromeda search engine<sup>19</sup>. Primary searches performed using a 6-ppm precursor ion tolerance when total proteins were analyzed. The MS/MS ion tolerance of 20 ppm was used. Carbamido-methylation of cysteine was specified as the control modification, and N-acetylation of protein and oxidation of methionine were considered as variable modifications. Enzyme specificity was set to full tryptic digestion. Peptides with a minimum length of six amino acids and up to two missed cleavages were included. The acceptable false discovery rate (FDR) was set to 1% at the peptide, protein, and modification levels. To maximize quantification events across samples, we enabled the 'Match between Runs' option of the MaxQuant platform. For label-free quantification, the Intensity Based Absolute quantification (iBAQ) algorithm<sup>16</sup> was used as part of the MaxQuant platform. The iBAQ values calculated by MaxQuant are the raw intensities divided by the number of theoretical observable peptides. Thus, the iBAQ values provide proportional to the molar quantities of the proteins.

### **Data processing for the DIA MS**

To generate the spectral libraries, 12 DDA measurements were performed on the urine samples. The DDA spectra were searched with the MaxQuant against the UniProt Human Database (December 2014, 88,657 entries) and the indexed retention time standard peptide sequence. A spectral library was generated using the spectral library generation feature in Spectronaut 10.

The DIA data from individual samples were analyzed with Spectronaut 10 (Biognosys, Schlieren, Switzerland). First, we converted the DIA raw files into the HTRMS format using the GTRMS converter tool provided with Spectronaut. The FDR was estimated with the mProphet<sup>20</sup> approach and set to 1% at the peptide precursor and protein levels. The proteins were inferred by the software, and the quantification information was acquired at the protein level using the q-value < 0.01 criterion, which was used for the subsequent analyses.

### **Study approval**

The Institutional Review Board at Seoul National University Children's Hospital approved the study, and written informed consent was obtained from the parents of participants prior to inclusion in the study. The research was performed in accordance with the Declaration of Helsinki (ethical principles for medical research involving human subjects). This trial has been registered at [www.clinicalTrials.gov](http://www.clinicalTrials.gov) (NCT02743273).

### **Statistical analyses**

Normally distributed continuous variables were compared using Student's t-test or Fisher's exact test. Nonnormally distributed continuous variables were analyzed using the Mann-Whitney U-test. Paired t tests and  $\chi^2$  tests were conducted to assess differences in laboratory measurements between the AKI and control groups. The results are expressed as the means with 95% CIs. Multiple logistic regression was conducted with the candidate biomarkers for the diagnosis of AKI after adjusting for gestational age. In the validation set, biomarkers were dichotomized at the values that optimized the sensitivity and specificity. To assess the predictive accuracy of the candidate biomarkers for the diagnosis of AKI, ROC curves were generated, and AUCs were computed with their CIs. All tests were 2-sided, with  $p < 0.05$



indicating statistical significance. All statistical analyses were performed with the IBM SPSS Statistics software package, version 24.0 (IBM, Armonk, NY).

### **Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Results

### Discovery phase

Among the 37 infants who were enrolled in the cohort study, 5 patients were identified as having AKI after ibuprofen treatment. The multiquantitative proteomics approach depicted in Fig. 1 was used to identify urinary proteins differentially expressed before and after ibuprofen administration in patients with AKI induced by ibuprofen treatment. In total, 1,810 proteins were identified (Supplementary Table S1), the distributions of the identified proteins according to the involved cellular components and biological processes are shown in Supplementary Fig. 1 and Supplementary Fig. 2. Among them, 349 unique proteins showed levels that differed by two-fold or more, with 107 changed in 4 patients and 67 changed in 5 patients. We selected 174 proteins that were either up- or down-regulated in AKI patients as the 1<sup>st</sup> targeted candidates: 55 up-regulated proteins and 52 down-regulated proteins in 4 patients, and 36 up-regulated proteins, and 31 down-regulated proteins in 5 patients were included (Fig. 2 and Supplementary Table S2).

### Verification phase

From the biorepository, sixteen urine samples from patients with AKI and 16 samples from controls that were matched by gestational age and postnatal age in the biorepository were selected and analyzed. We performed DIA-based targeted proteomics using Spectronaut software. We were able to quantify 168 of the 174 proteins identified in the discovery phase (Supplementary table S3). Among them, the levels of only six proteins were significantly different, and these proteins were all up-regulated in the AKI group compared to the control group. NGAL, cartilage intermediate layer protein 2, 6-PGLS, annexin A5, galectin 3, and

protein S100-P were finally selected as final candidate urinary AKI biomarkers.

### **Validation phase**

To evaluate the predictive value of the 6 urinary biomarkers for AKI, we selected additional patients who developed AKI within 7 days after birth without any postnatal insults and those who did not develop AKI within 7 days after birth for the control group from the repository and matched them according to gestational age. Samples of the first-day urine collected within 24 hours after birth were also analyzed. Fourteen infants who were identified as having AKI within 7 days after birth and 14 infants matched by gestational age without AKI were selected. Prenatal, clinical, and laboratory characteristics are listed in Table 1. Infants with AKI had lower 1- and 5-minute Apgar scores. There were no differences in the perinatal characteristics, including preeclampsia and oligohydramnios. Of those with AKI, 4 of 14 (28.6%) died, compared with 1 of 14 (7.1%) of those without AKI. In the AKI group, 6 patients developed chronic kidney disease after discharge.

Six final candidate urinary biomarkers were measured with ELISAs in 28 samples of the first-day urine. The levels of each biomarker were compared between infants with AKI and those without AKI, as shown in Table 2. Among the 6 urinary biomarkers, CILP-2 was not compared between the AKI patients and controls because of the very low levels in both groups. NGAL was significantly elevated in the AKI group compared with that in the control group (44.53 ng/mL, 95% confidence interval (CI) 34.52-51.41 vs. 25.18 ng/mL, 95% CI 11.16-38.02;  $p = 0.008$ ). Similarly, annexin A5 (1877.11 pg/mL, 95% CI 689.43-2874.79 vs. 320.65 pg/mL, 95% CI 232.13-457.86;  $p = 0.006$ ), 6-PGLS (95.81 ng/mL, 95% CI 79.32-105.20 vs. 70.39 ng/mL, 95% CI 47.63-92.56;  $p = 0.023$ ), and protein S100-P (434.88 pg/mL, 95% CI 187.44-674.55 vs. 146.86 pg/mL, 95% CI 50.80-242.93;  $p = 0.019$ ) were significantly elevated in the AKI

group compared with their levels in the control group (Figure 3).

For every 10 ng/mL increase in the concentration of NGAL, the odds of having AKI increased by 79% (OR = 1.79, 95% CI 1.12-3.05; p=0.018). For the same increase in the concentration of PGLS, the odds increased by 38% (OR = 1.38, 95% CI 1.02-1.89; p=0.035). For every 100 pg/mL increase in the level of annexin A5, the odds of having AKI increased by 82% (OR = 1.82, 95% CI 1.11-2.99; p=0.031).

The maximum values of these biomarkers were well able to predict AKI; the areas under the curve (AUCs) were 0.75 for NGAL, 0.882 for annexin A5, 0.748 for protein S100-P and 0.667 for 6-PGLS (Table 2). Combining NGAL and annexin A5 in one model improved the ability to detect AKI (AUC = 0.923). Combining NGAL, annexin A5, and protein S100-P increased the AUC value to 0.932 (Figure 4).

The derived sensitivities, specificities, and predictive values at the determined cut-off concentrations for the urinary biomarkers are listed in Table 3.

**Table 1.** Basal characteristics for the validation set

|  | <b>AKI</b>        | <b>No AKI</b>      | <b>p-value</b> |
|--|-------------------|--------------------|----------------|
|  | <b>(n=14)</b>     | <b>(n=14)</b>      |                |
| <b>Patient demographics</b>                    |                   |                    |                |
| Gestational age (week), mean $\pm$ SD          | 27.6 $\pm$ 2.6    | 27.9 $\pm$ 2.6     | 0.8            |
| Birth weight (g), mean $\pm$ SD                | 905.1 $\pm$ 399.1 | 1070.0 $\pm$ 296.5 | 0.282          |
| Male, n (%)                                    | 5 (35.7)          | 7 (50)             | 0.704          |
| Small for gestation (<10 percentile), n (%)    | 3 (21.4)          | 1 (7.1)            | 0.596          |
| Apgar score at 1 minute, median (min, max)     | 2 (0-6)           | 4 (1-7)            | 0.016          |
| Apgar score at 5 minutes, median (min, max)    | 5 (2-7)           | 7 (5-10)           | 0.004          |
| Mortality, n (%)                               | 4 (28.6)          | 1 (7.1)            | 0.326          |
| Chronic kidney disease, n (%)                  | 6 (42.9)          | 1 (7.1)            | 0.077          |
| <b>Prenatal characteristics</b>                |                   |                    |                |
| Antenatal steroid, n (%)                       | 8 (57.1)          | 10 (71.4)          | 0.43           |
| Maternal preeclampsia, n (%)                   | 0 (0)             | 1 (7.1)            | 1              |
| PROM, n (%)                                    | 6 (42.9)          | 7 (50)             | 0.705          |
| Histologic chorioamnionitis, n (%)             | 6 (42.9)          | 5 (35.7)           | 0.699          |
| Maternal kidney disease, n (%)                 | 0 (0)             | 1 (7.1)            | 1              |
| Oligohydramnios, n (%)                         | 4 (28.6)          | 0 (0)              | 0.098          |
| Diabetes mellitus, n (%)                       | 0 (0)             | 0 (0)              | 1              |
| <b>Laboratory measurements (within 7 days)</b> |                   |                    |                |
| SCr at cord blood (mg/dL), mean $\pm$ SD       | 0.38 $\pm$ 0.18   | 0.49 $\pm$ 0.17    | 0.089          |
| SCr at PND 3 days (mg/dL), mean $\pm$ SD       | 1.64 $\pm$ 0.59   | 0.77 $\pm$ 0.14    | <0.001         |

|  |                 |                  |        |
|--|-----------------|------------------|--------|
| SCr at PND 7 days (mg/dL), mean $\pm$ SD                             | 2.05 $\pm$ 0.55 | 0.67 $\pm$ 0.18  | <0.001 |
| $\Delta$ SCr between PND 3 days and 7 days<br>(mg/dL), mean $\pm$ SD | 0.41 $\pm$ 0.36 | -0.09 $\pm$ 0.13 | <0.001 |

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AKI, acute kidney injury; PROM, premature rupture of membrane; SCr, serum creatinine; PND, postnatal day

**Table 2.** Urinary biomarkers according to acute kidney injury status

| <b>Protein</b>           | <b>AKI (n=14)</b>            | <b>Control (n=14)</b>      | <b>p-value</b> | <b>AUC</b> |
|--------------------------|------------------------------|----------------------------|----------------|------------|
| NGAL, ng/mL              | 44.53<br>(34.52, 51.41)      | 25.18<br>(11.16, 38.02)    | 0.008          | 0.75       |
| Annexin A5, pg/mL        | 1877.11<br>(689.43, 2874.79) | 320.65<br>(232.13, 457.86) | 0.006          | 0.882      |
| Galectin 3, ng/mL        | 16.49<br>(9.30, 20.84)       | 16.77<br>(12.26, 21.06)    | 0.929          | 0.472      |
| 6-PGLS, ng/mL            | 96.81<br>(79.32, 105.20)     | 70.39<br>(47.63, 92.56)    | 0.023          | 0.667      |
| Protein S100-P,<br>pg/mL | 434.88<br>(187.44, 674.55)   | 146.86<br>(50.80, 242.93)  | 0.019          | 0.748      |

NGAL, neutrophil gelatinase-associated lipocalin; 6-PGLS, 6-phosphogluconolactonase;

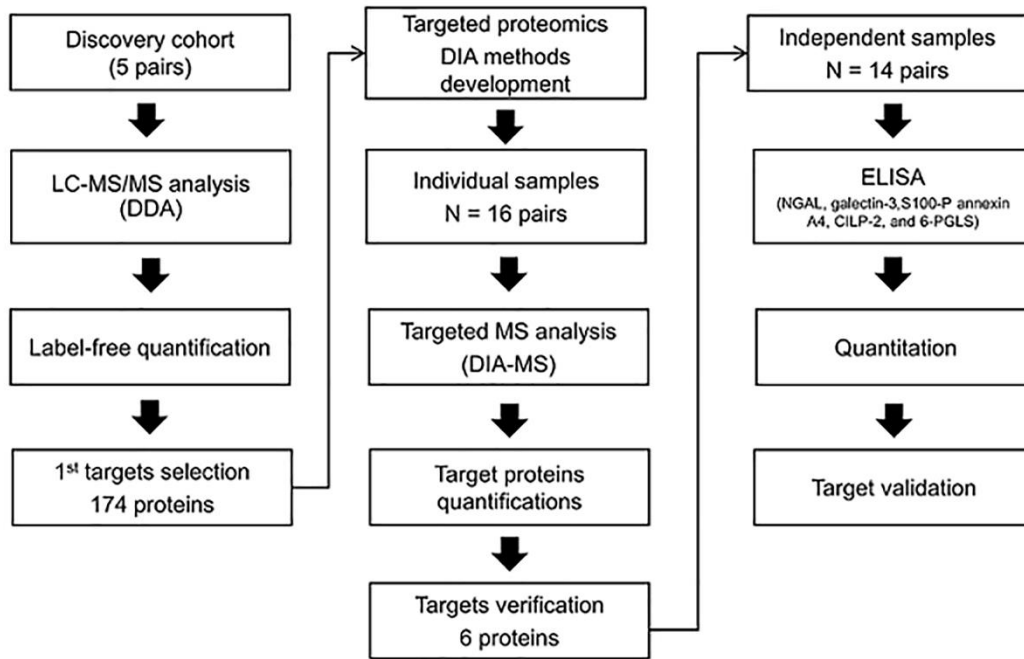
AKI, acute kidney injury

**Table 3.** Urinary biomarker characteristics for prediction of AKI

| <b>Biomarker</b>       | <b>Cut-off</b> | <b>Sensitivity</b> | <b>Specificity</b> | <b>PPV</b> | <b>NPV</b> |
|------------------------|----------------|--------------------|--------------------|------------|------------|
| NGAL (ng/mL)           | 42.15          | 0.71               | 0.71               | 0.71       | 0.71       |
| Annexin A5 (pg/mL)     | 409.86         | 0.85               | 0.71               | 0.73       | 0.83       |
| Protein S100-P (pg/mL) | 110.70         | 0.83               | 0.64               | 0.77       | 0.78       |

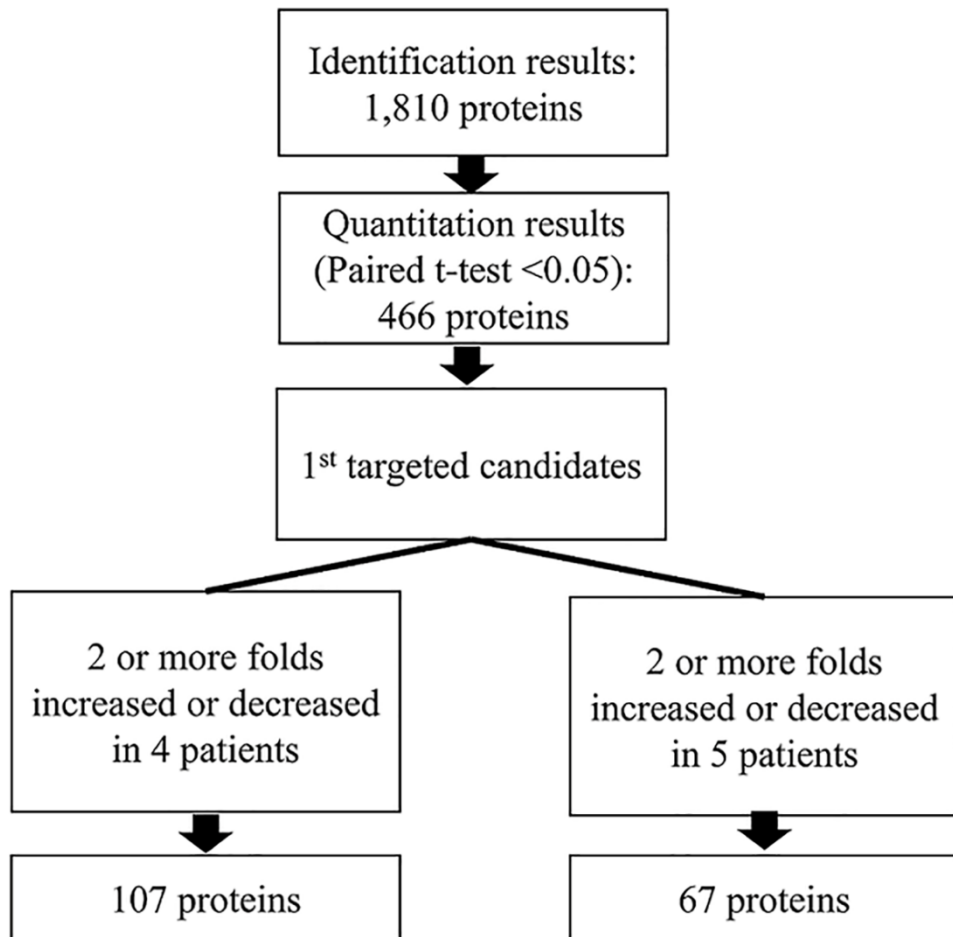
NGAL, neutrophil gelatinase-associated lipocalin; PPV, positive predictive value; NPV, negative predictive value



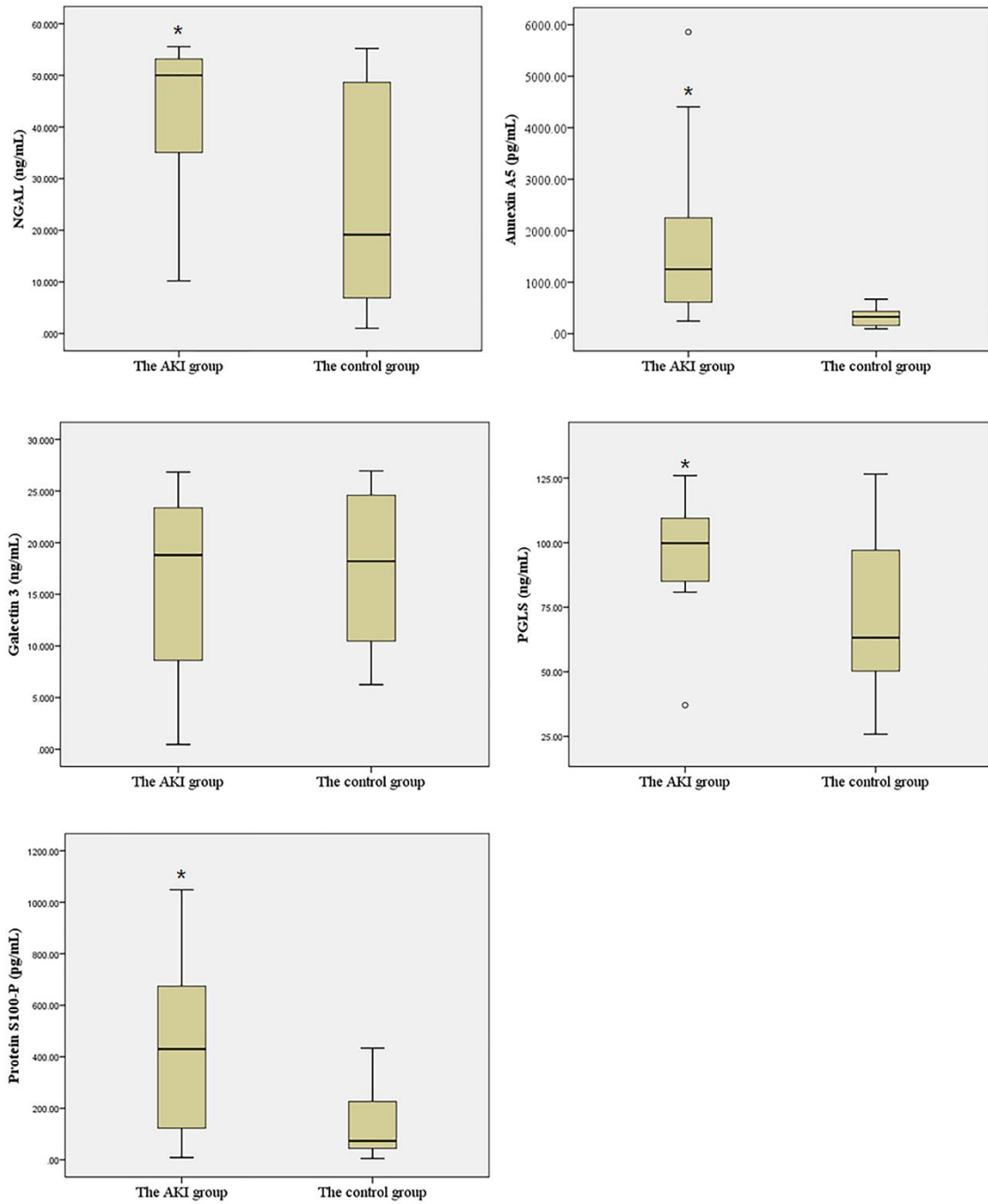


**Fig. 1.** Work flow in proteomic biomarker discovery experiments.

LC MS/MS, liquid chromatography-tandem mass spectrometry; DDA, data-dependent acquisition; DIA, data-independent acquisition; ELISA, enzyme-linked immunosorbent assays

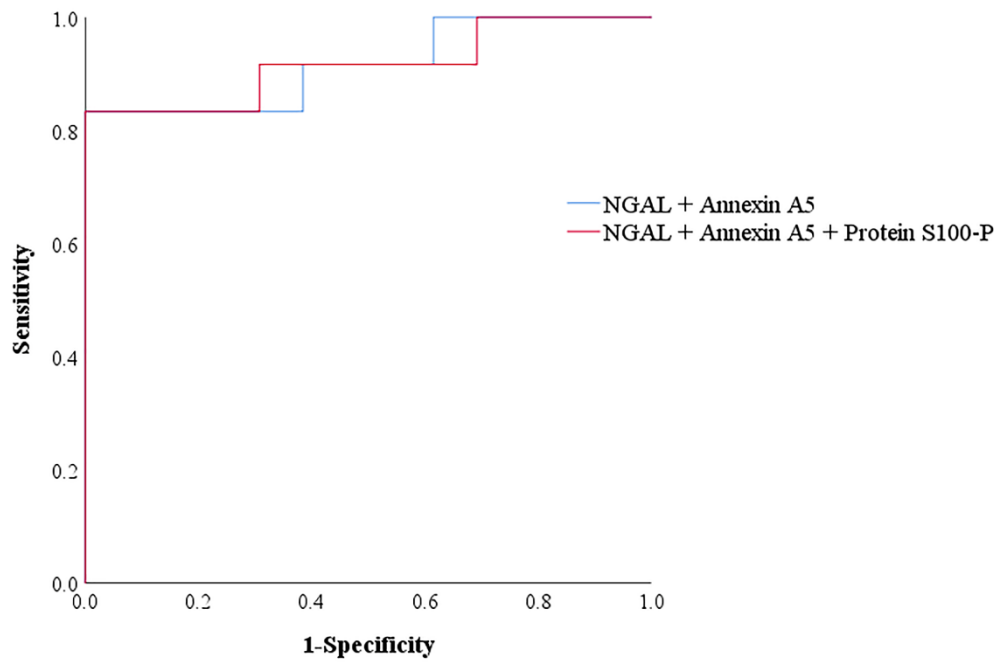


**Fig. 2.** Schematic representation of the target protein selection process in the discovery phase.

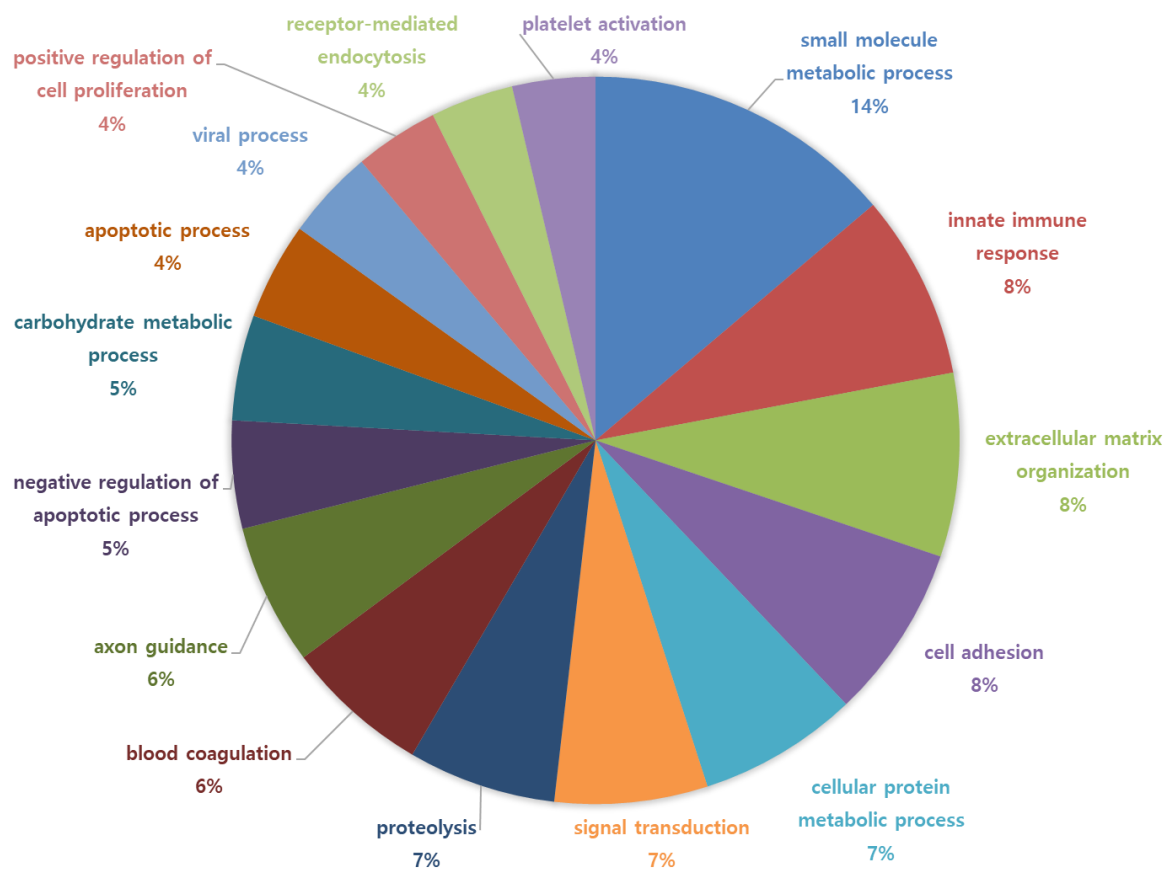


**Fig. 3.** Box and whisker plots of urine values of NGAL, annexin A5, S100P, 6-PGLS, and galectin 3 in preterm infants with AKI and infants without AKI during the first day of life.

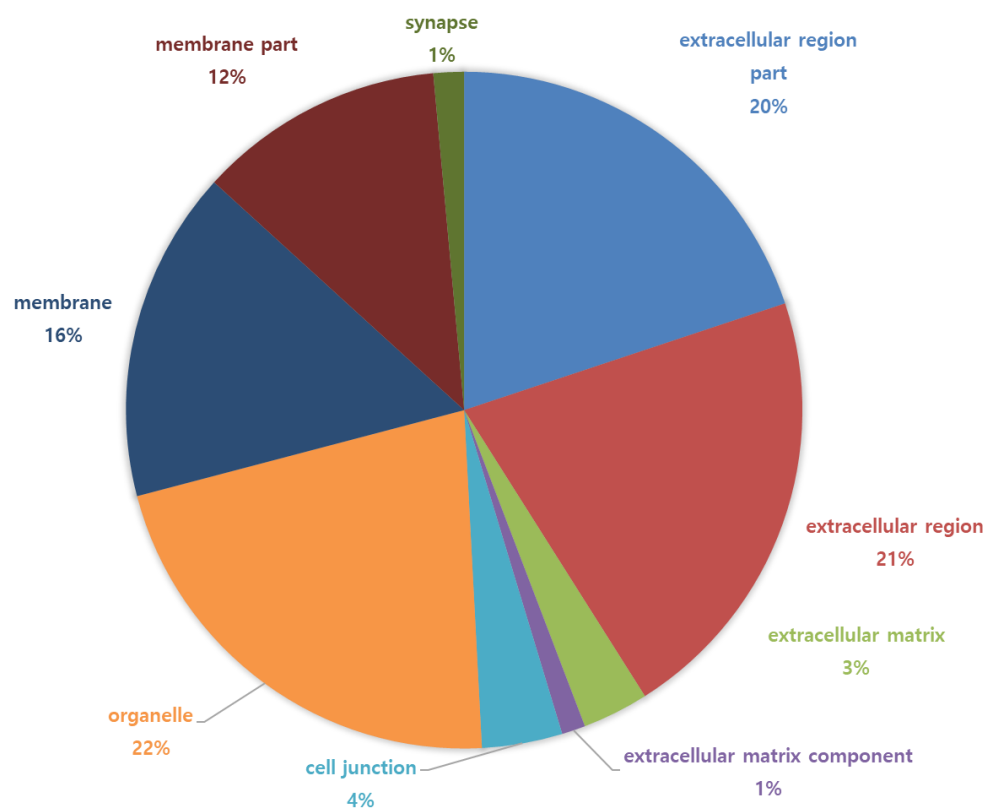
\* p-value < 0.05, data are presented as box and whisker plots



**Fig. 4.** ROC AUC values for combined urinary biomarkers; their predictive abilities to detect AKI in preterm infants.



**Supplementary Fig. S1.** Distribution of the proteins identified according to cellular components.



**Supplementary Fig. S2.** Distribution of the proteins identified according to biological processes.

## Discussion

To the best of our knowledge, this is the first proteomic study to identify novel urinary AKI biomarkers in preterm infants.

This study described the identification, verification and validation of urinary biomarkers for AKI in extremely preterm infants. NGAL, annexin A5, and protein S100-P showed excellent abilities to differentiate between patients who developed AKI within 7 days after birth and those who did not. We used samples of the first-day urine, which were collected within 24 hours after birth, and these biomarkers showed good prognostic potential, with increased concentrations of the biomarkers associated with the development of AKI. When the accuracy was assessed using receiver operating characteristic (ROC) curves, the combination of NGAL and annexin A5, as well as NGAL, annexin A5, and protein S100-P, performed well.

Urine NGAL is postulated to be a highly sensitive marker of AKI, specifically of tubular cell damage rather than a decrease in glomerular filtration<sup>21</sup>. Urinary NGAL levels are strongly correlated with SCr levels, and this identification preceded that of traditional markers in critically ill pediatric and adult populations. However, only a few studies have analyzed urine NGAL levels in preterm infants. In one such study, urine NGAL concentrations in preterm infants showed low sensitivity for the detection of renal impairment and appeared variable in preterm populations<sup>10</sup>. Suchojad et al. also demonstrated that inflammatory status and immaturity limit the specificity of urine NGAL as a marker of AKI<sup>22</sup>. In contrast, we showed that urine NGAL levels obtained during the first day of life can predict AKI with a sensitivity and specificity of 0.71.

There is a lack of data supporting the use of S100P and annexin A5 as biomarkers for

predicting AKI. However, many vital physiological functions and metabolic processes are regulated by  $\text{Ca}^{2+}$ , and a large number of human diseases are linked to altered  $\text{Ca}^{2+}$  homeostasis.  $\text{Ca}^{2+}$  signals within cells are transmitted by many  $\text{Ca}^{2+}$  binding proteins, including the S100 protein family and annexins.

The S100 proteins are the largest subgroup within the superfamily of EF-hand  $\text{Ca}^{2+}$  - binding proteins. S100 proteins have been investigated because of their close associations with human disease, including cancer, neurodegenerative disorders, and cardiomyopathies, and their recent use in diagnosing disease. S100P, a member of the S100 protein family, was initially identified in the placenta, and its widespread expression was later noted in the gastrointestinal tract, prostate, leukocytes, and renal pelvic urothelium<sup>23</sup>. S100P is known to be expressed in several malignant neoplasms, especially renal neoplasms<sup>24</sup>. Although we identified that S100P could be a novel biomarker for AKI in the present study, there have been no previous studies on the role and expression of S100P during the development of the kidneys during the fetal period. Therefore, further studies are needed to investigate the association between S100P and kidney injury, including underlying biological pathophysiology.

Annexins are also  $\text{Ca}^{2+}$  and phospholipid-binding proteins forming an evolutionarily conserved multigene family. Some annexins have been considered to participate in the regulation of membrane organization and trafficking, as well as the regulation of ion currents across membranes<sup>25,26</sup>. In addition, some members of this family have been identified as receptors for endothelial proteases and inhibitors of neutrophil migration and blood coagulation. Deregulation of annexin expression and activity, which might have stress related functions, has been associated with human disease. Among the annexin family, Annexin A5 has been found to be upregulated by changes in the cellular hypoxic



state, and identified as a marker of apoptotic changes induced by hypoxic stress<sup>27,28,29</sup>. As ischemic renal dysfunction significantly contributes to apoptosis, annexin A5 can be a biomarker for predicting AKI. Regarding the lack of studies on these biomarkers, which were first discovered in the present study, further studies would be helpful for understanding the precise functions and pathophysiological mechanisms of individual biomarkers.

To date, several studies on biomarkers of kidney injury have investigated multiple markers capable of the early detection of structural injury that is also associated with outcomes. There are a few promising urinary biomarkers, such as NGAL, IL-18, and KIM-1, that can be representative sequential biomarkers that would be useful for determining the timing of the initial insult and assessing the duration of AKI<sup>30</sup>. Although these results are promising, individual biomarkers still lack sensitivity and specificity. Biomarkers with an AUC value of 0.5 are not better than expected by random chance, and a biomarker with an AUC of approximately 0.75 is generally considered to be a good biomarker. In this study, we demonstrated that the AUC value for combining urinary NGAL, S100P, and annexin A5 was 0.932, which represents an excellent biomarker.

This study has several strengths. First, we conducted a prospective cohort study, which allowed the data to be collected consistently, and all factors were taken into account in the study design and the data analysis and interpretation. Different samples were used in each stage, and the candidate biomarkers were ultimately confirmed using conventional immunoassays. Second, several previous studies have shown that the urinary concentrations of some biomarkers are dependent on gestational age in neonates without AKI<sup>31</sup>. This may be secondary to the inability of immature tubules to reabsorb these proteins in underdeveloped kidneys. Therefore, controlling for gestational age is

necessary when determining the associations between urinary biomarkers and AKI. In this study, we selected urine samples from gestational age- and postnatal age-matched neonates for the control group. Third, we did not use urine creatinine concentration because this can also reflect kidney injury. Instead of calibrating the urinary creatinine level in all urine samples, the amounts of protein in each sample were adjusted to the same value in this study<sup>32</sup>. Forth, for identification and verification of urinary biomarkers for AKI, we analyzed urine samples that met the definition of AKI as an increase in SCr. Instead, when validating the biomarkers, we used samples of the first-day urine before the increase in SCr, which likely reflects prenatal kidney function in preterm infants. Premature infants have with a very low glomerular filtration rate; therefore, mild exposure to nephrotoxic agents can cause a high degree of injury<sup>33</sup>. However, premature infants are at significantly elevated risks for sepsis and exposure to nephrotoxic medications such as antibiotics and COX inhibitors. Additionally, most preterm infants who develop AKI are extremely premature and develop AKI within the first week after birth<sup>3</sup>. Of note, neonatal serum biomarkers such as Cr during the first few days of life may reflect the mother's kidney function but not their own. The advantage of using the first-day urine after birth is that we can directly evaluate the potential risk of AKI by estimating underlying prenatal kidney insults induced by prenatal pathological conditions and perinatal insults and can ameliorate AKI by preventing modifiable risk factors.

This study has some possible limitations because of the small sample size. After the discovery and initial validation of biomarkers, further large-scale studies are needed to confirm their clinical utility. With regard to the clinical application of these urinary biomarkers, the estimation of the amount of damage for risk stratification and prediction of disease progression and clinical outcomes should also be performed.

## **Conclusion**

This study provides new insights into the potential role of proteomics in the identification of novel urinary AKI biomarkers in premature infants. According to our results, NGAL, S100P, and annexin A5 can be early biomarkers for AKI before the increase in SCr in newly born infants. In addition, the combinations of NGAL, S100P, and annexin significantly improved the ability to predict AKI. The early detection of kidney injury can be helpful clinically for neonatologists in the prevention of further postnatal kidney insults.

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## 요약 (국문초록)

### 단백체학을 이용한 미숙아의 급성 신손상의 조기 소변 생체지표의 개발

#### 목적

최근 의료기술의 현저한 발전으로 인해 극소저출생체중아의 생존률은 현저히 높아지면서, 미숙아의 생존률과 함께 장기적 예후에 대한 관심이 높아지고 있다. 특히 태아의 발달 과정중에 태어난 미숙아는 여러 발달 단계의 손상 및 정체를 겪게 되며, 이는 장기적으로 성인기의 여러 만성 질환 발생 및 대사 증후군의 위험도를 증가시킨다는 연구들이 발표되고 있다. 미숙아에서 급성 신손상의 발생률은 약 8-24%의 비율로 발생한다고 보고하고 있으며, 특히 출생 체중이 1500 g 미만의 극소저출생체중아에서의 급성 신손상의 발생률은 20-30%로 높은 비율을 차지한다고 알려져 있다. 이러한 신장의 손상은 미숙아의 단기 사망률 및 여러 질환의 이환율 증가에 영향을 주며, 생존한 미숙아의 장기적 예후에도 나쁜 영향을 주는 것으로 알려져 있다. 따라서, 미숙아에서 신장 기능의 손상을 조기에 발견하고 모니터링 하는 것은 이후 반복되는 신장 손상을 예방하고 추적관찰 함으로써 장기적 예후를 호전시키는데 도움이 될 수 있을 것이다. 이에 본 연구는 Proteomics를 이용하여 미숙아의 급성 신손상을 예측할 수 있는 urinary biomarker를 찾아, 미숙아의 급성 신손상을 조기에 발견하고자 하며 이에 대한 임상적인 유용성을 확인하고자 한다.

## 방법

본 연구는 총 3단계로 진행한다.

1. Discovery phase: 재태 주수 32주 미만 혹은 출생 체중 1500g 미만의 미숙아로 동맥관 개존증으로 ibuprofen약물 사용이 결정되는 경우, 약물 투여 전과 후의 약 6시간씩 소변을 전향적으로 수집한다. ibuprofen 투여 후 신손상이 확인된 환자들의 소변을 대상으로 ibuprofen 투여 전과 후 소변을 label-free mass spectrometry (MS) based protein quantification을 이용하여 분석하여 이중 의미 있게 변화하는 단백질을 candidate biomarker로 선정한다.

2. Verification phase: 재태 주수 32주 미만 혹은 출생 체중 1500g 미만의 미숙아를 대상으로 서울대학교병원 신생아중환자실에서 진행하는 “신생아중환자실에 입원한 신생아의 임상적 database수집과 생물학적 검체 은행 구축” 연구에 동의하여 보관된 소변 검체를 이용하여, 급성 신손상이 발생한 미숙아의 소변과, 주수와 출생 후 나이를 matching한 대조군 소변을 골라 discovery phase에서 확인된 candidate biomarker를 target으로 data-independent acquisition (DIA) method를 통해 분석한다.

3. Validation phase: 서울대학교병원 신생아중환자실에서 진행하는 검체 은행 구축 연구에 동의하여 보관된 소변 검체를 이용하여, 출생 7일 이내 급성 신손상이 발생한 환자의 첫 소변 검체와, 주수를 matching한 대조군의 출생 첫 소변 검체를 이용하여 biomarker가 출생 첫 날부터 의미 있게 증가하여 임상적 유용성이 있는지를 enzyme-linked immunosorbent assay (ELISA)를 이용하여 분석한다.

## 결과

총 1,810개의 단백질이 discovery phase에서 발견되었으며, 그 중 349개의 단백질이 2배 혹은 그 이상의 변화를 보였다. 이 중, discovery phase의 대상자인 5명의 환자 중 4명에서 2배 이상의 증가 혹은 감소의 변화를 보인 107의 단백질과, 5명 모두에서 2배 이상의 변화를 보인 67개의 단백질을 1<sup>st</sup> targeted candidate로 선정하였다. 이를 이용하여 총 16 명의 급성 신손상을 보인 환자의 소변 검체와, 주수와 출생 후 나이를 매칭한 대조군 16명의 소변 검체를 이용하여 verification 을 진행하였다. 총 174개의 1<sup>st</sup> targeted candidates 단백질 중에서 168개만이 분석되었고, 이중 총 6개의 단백질인 Neutrophil gelatinase-associated lipocalin (NGAL), cartilage intermediate layer protein 2 (CLIP-2), 6-phosphogluconolactonase (6-PGLS), annexin A5, galectin 3, protein S100-P가 통계적으로 의미 있게 차이가 있음을 확인되어 최종 후보 단백질로 선정되었다. 이를 대상으로 총 14쌍의 환자와 대조군의 생후 첫 소변 검체에서 의미 있는 차이를 보이는지 ELISA를 이용하여 validation을 시행하였으며, 6개의 후보 단백질 중 NGAL, annexin A5, 6-PGLS, protein S100-P가 환자군에서 의미 있게 증가함을 확인하였다. 급성 신손상을 예측하는 능력을 보기 위해 the area under the curve (AUCs)를 확인하였으며, 최종 NGAL, annexin A5, protein S100-P의 조합이 AUC 0.932의 높은 예측력을 보였다.

## **결론**

NGAL, protein S100-P, annexin A5가 출생 직후 급성 신손상의 위험을 예측하는

biomarker로 확인되었다. 본 연구를 통해 proteomic을 이용한 급성 신손상을 예측하기 위한 새로운 urinary biomarker를 발견하였으며, 이를 통해 미숙아의 신손상을 조기에 예측하고 출생 후 추가적인 신손상을 예방하여 예후를 향상시키는데 도움을 줄 수 있을 것이다

**주요어:** 미숙아, 급성 신손상, proteomics, biomarker

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