Recent advances in electron microscopy for the diagnosis and research of glomerular diseases

Diminazene aceturate exacerbates renal fibrosis after unilateral ureteral obstruction in female mice

Upregulation of NADH/NADPH oxidase 4 by angiotensin II induces podocyte apoptosis

Evaluating the Safety and effectiveness in adult KorEaN patients treated with Tolvaptan for management of autosomal dominAnt polycystic kidney disease (ESSENTIAL): short-term outcomes during the titration period

Serum calcification propensity and its association with biochemical parameters and bone mineral density in hemodialysis patients
Aims and Scope

Kidney Research and Clinical Practice (KRCP; formerly The Korean Journal of Nephrology; ISSN 1975-9460, launched in 1982), the official journal of the Korean Society of Nephrology, is an international, peer-reviewed journal published in English. Its ISO abbreviation is Kidney Res Clin Pract.

The journal considers articles on all aspects of nephrology and hypertension as well as molecular genetics, anatomy, pathology, physiology, pharmacology, and immunology related to kidney disease. In particular, the journal focuses on translational renal research that helps bridging laboratory discovery with the diagnosis and treatment of human kidney disease. The journal publishes the topics covered basic science with possible clinical applicability and the papers on the pathophysiological basis of the kidney disease. Original studies from areas of diagnostic and interventional nephrology or dialysis access are also welcomed. Major article types considered for publication include original research and reviews on current topics of interest.

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The image on the front cover: Honda et al showed the scanning transmission electron microscopy (STEM) images. STEM technology enables the performance of high resolution analysis. Please see the text for more details (pp. 155-65).
Updates in renal pathology

Hyeon Joo Jeong

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Our understanding on the pathophysiology of glomerular diseases has largely relied on meticulous evaluation and strategic approaches to renal biopsy material using light microscopy (LM), immunofluorescence (IF), and electron microscopy (EM). Glomerular morphology has been categorized based on injury patterns indicated by changes in glomerular cellularity and cell types, and abnormal loop changes under LM, after which several primary glomerular diseases have been named, such as focal segmental glomerulosclerosis and diffuse proliferative or mesangio-proliferative glomerulonephritis. Subtyping of glomerular morphology has been used as a therapeutic guide or prognostic parameter of immunoglobulin A (IgA) nephropathy, lupus nephritis, and antineutrophil cytoplasmic antibody-associated glomerulonephritis. IF can be used to identify IgA nephropathy and C3 glomerulopathy regardless glomerular histology, meanwhile diffuse glomerular basement membrane (GBM) thinning on EM characterizes thin GBM nephropathy. Recently, immunohistochemistry, mass spectrometry, and next-generation sequencing techniques have been included in the diagnosis of fibrillary glomerulonephritis, typing of amyloid fibrils, and exploring the etiology of hereditary nephropathy, respectively. We admit that correlations between renal morphology and clinical findings continue to fall short of our expectations, and we hope that further investigations into glomerular morphology and the discovery of new diagnostic/research tools will narrow this gap.

This special issue of *Kidney Research and Clinical Practice* focuses on two selected topics, an update on lupus nephritis (glomerular morphology) and a three-dimensional EM technique (new diagnostic/research tool).

The first World Health Organization (WHO) classification of lupus nephritis was formulated in 1974, and modified in 1982, yet the benefit of renal biopsy in the prediction of renal outcomes and as a prognostic indicator remains an issue [1]. The 2003 International Society of Nephrology (ISN)/Renal Pathology Society (RPS) classification was another modified WHO system, which was more complex than the previous systems, did not reach a consensus among renal pathologists, and did not significantly improve clinicopathological correlation [1–3]. The modified ISN/RPS classification was proposed in 2018, of which classes were simplified and histologic indices were added [4]. Choi et al. [5] reviewed the 2018 ISN/RPS classification, especially the activity and chronicity indices, which were modified from the National Institutes of Health-sponsored 1983 classification, and the clinical significance of these histologic indices.

Conventional transmission EM (TEM) is useful for identifying glomerular cellular and GBM alterations, but is limited in viewing the whole scope of changes due to its two-dimensional nature. To overcome this limitation, Honeda et al. [6] introduced several three-dimensional EM tech-
nologies—three-dimensional EM, correlative light and EM, low vacuum SEM, and scanning TEM—and demonstrated possible clinical applications in select glomerular diseases [7–12]. These techniques are fascinating in that glomerular podocytes and GBM can be observed three-dimensionally in frozen or formalin-fixed paraffin-embedded sections, and that the area of interest on LM and IF can be correlated with ultrastructural features.

I hope the concise, well-summarized reviews of lupus nephritis and EM methods in this special issue will contribute up-to-date information and knowledge that can be used in future patient management and research on glomerulonephritis.

Conflicts of interest

The author has no conflicts of interest to declare.

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References

Recent advances in electron microscopy for the diagnosis and research of glomerular diseases

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²Division of Electron Microscopy, Showa University, Tokyo, Japan

Recent technical advances in the detection of backscattered electrons during scanning electron microscopy (SEM) have improved resolution and have provided several new technologies for research and clinical practice in kidney disease. The advances include three-dimensional (3D) electron microscopy (3D-EM), correlative light and electron microscopy (CLEM), low-vacuum SEM (LVSEM), and scanning transmission electron microscopy (STEM). 3D-EM analysis used to be laborious, but recently three different technologies, serial block-face SEM, focused ion beam SEM, and array tomography, have made 3D-EM easier by automating sectioning and the subsequent image acquisition in an SEM. CLEM is a method to correlate light microscopic images, especially immunofluorescent and electron microscopy images, providing detailed ultrastructure of the area of interest where the immunofluorescent marker is located. LVSEM enables the use of SEM on materials with poor electron conductivity. For example, LVSEM makes it possible for high resolution, 3D observation of paraffin sections. Finally, STEM is a method to observe ultrathin sections with improved resolution by using the focused electron beam scanning used in SEM and not the broad electron beam used in transmission electron microscopy. These technical advances in electron microscopy are promising to provide plenty of novel insights for understanding the pathogenesis and diagnosis of various glomerular diseases.

Keywords: Array tomography, Backscattered electrons, Correlative light and electron microscopy, Focused ion beam, Low-vacuum scanning electron microscopy, Scanning transmission electron microscopy, Serial block-face

Introduction

There are two kinds of electron microscopy (EM); transmission EM (TEM) and scanning EM (SEM). TEM has been used for routine examination for kidney biopsy diagnosis, and vast knowledge of various glomerular diseases has been accumulated. The advantage of TEM is its high resolution made possible by the high acceleration voltage (60–200 kV) of its electron beam. In TEM applied to biological specimens, the usual acceleration voltage is 100 kV, which can penetrate the sample to a depth of 100 nm. That is deeper than the usual thickness of ultrathin sections (60–80 nm) and can enable a resolution of 1 nm. Conversely, in an SEM the acceleration voltage is 10 to 30 kV to avoid excessive penetration of the electron beam because the purpose of SEM is to view the surface. SEM acquires surface information by detecting two different kinds of electrons; secondary electrons (SE) and backscattered electrons (BSE).
When an electron beam reaches the sample, SE are generated from a superficial zone of the sample with a depth of up to 10 nm, reflecting the surface property of the sample. In contrast, BSE are generated from sample depths on the order of several tens of nm, revealing the elemental composition and density of the sample (Fig. 1). Elements with higher atomic number generate stronger BSE.

Because the amount of BSE is relatively low from the low acceleration voltage of 10 to 30 kV in SEM, BSE used to be difficult to detect, making it challenging to determine surface information. In 1996, Richards and ap Gwynn [1] reported a novel method for viewing the surface of the resin block by detecting BSE in SEM, which promoted the use of SEM. Because of its ease of sample preparation and the lack of a need for ultrathin sectioning, SEM is easier to use compared to TEM. Recently, the improved ability to detect BSE for surface imaging has further extended the utility of SEM, suggesting that SEM may replace TEM for routine EM examinations in the future [2].

Three-dimensional electron microscopy analysis by scanning electron microscopy (volume scanning electron microscopy)

For a better understanding of morphological architecture, a three-dimensional (3D) image is undoubtedly superior to a two-dimensional one. A 3D observation of a cell or tissue can be achieved with considerably high resolution using confocal laser scanning microscopy (CLSM). However, to get further detailed ultrastructural information, EM analysis is required. Although SEM can easily obtain 3D images of the surface, the internal ultrastructure, such as neuronal

Figure 1. Backscattered electrons (BSE) and secondary electrons (SE) of scanning electron microscopy. The emitted electron (primary electron [PE]) beam generates three kinds of electrons after reaching the sample: transmission electrons (TE), SE, and BSE. TE are affected by the elemental composition and density of the sample and are detected at the detection plate under the sample in TE microscopy (TEM). SE are generated at the surface and affected by the surface irregularities and contours of the sample surface to a depth of up to 10 nm. BSE, also called posterior scattered electrons, are derived from the surface layer consisting of several tens of nanometers nm and are affected by the elemental composition, density, and concave-convex nature of the surface. BSE intensity depends on the atomic number and the acceleration voltage, which influences the depth of penetration of the PE into the sample. Thus, the acceleration voltage should be properly adjusted to acquire the surface information of the sample.
circuits or cell organelles, cannot be observed by SEM. In addition, the resolution capacity of SEM is also limited compared to TEM. Therefore, acquisition and reconstruction of serial TEM images are necessary to perform 3D ultrastructural analysis at high resolution. The generation and analysis of serial TEM sections for 3D reconstruction requires a highly skilled technique along with a lot of time and labor. These technical obstacles have been overcome by recent advances in automatic serial sectioning and imaging by SEM. Currently, there are three different procedures for 3D ultrastructure imaging: serial block-face (SBF) SEM, focused ion beam (FIB) SEM, and array tomography (Fig. 2) [3–5]. The methods for sample preparation to make a resin-embedded block and the analytical procedures after acquiring the serial image data are common in all three procedures. The characteristics of each procedure, including advantages, disadvantages, and suitable objects for the research, are discussed below and summarized in Table 1.

**Serial block-face scanning electron microscopy**

SBF-SEM requires an ultramicrotome installed inside the SEM vacuum chamber. An ultrathin section is cut with a diamond knife, and the exposed surface of the sample is scanned with an electron beam and detected by BSE to capture a TEM-like image. Serial images of the sample can be obtained consecutively, repeating this procedure (Fig. 2A). In this method, the specimen size is usually limited to...
0.5 mm, depending on the width of the diamond knife, and the acceleration voltage is restricted to 1–10 kV to avoid damage to the tissue of the next surface sectioned, which limits the system’s ability to achieve high resolutions. The sample size can be up to 1 mm in width, which is as much as the ultramicrotome can cut. Another disadvantage of SBF-SEM is the loss of cut sections, making it impossible to perform further examinations.

Ichimura et al. [6] investigated the 3D ultrastructure of podocytes using SBF-SEM and revealed that a novel structure of “ridge-like prominences” formed on the basal side of the podocyte cell body and was the primary platform for projecting foot processes. Randles et al. [7] also revealed 3D ultrastructural alterations of podocytes and the glomerular basement membrane (GBM) in a variety of mouse models of human glomerular diseases, such as Alport syndrome, focal segmental glomerulosclerosis, and childhood-onset nephrotic syndrome. Takaki et al. [8] demonstrated 3D morphological alterations of podocytes and mesangial cells in kidney biopsy samples of lupus nephritis and revealed multiple direct contacts of podocyte cytoplasmic processes with the mesangial cell body (Fig. 3). Nagai et al. [9] also demonstrated a similar phenomenon in which podocytes and mesangial cells established intercellular contacts through disrupted GBM in the glomerulus of immunoglobulin A (IgA) nephropathy.

### Focused ion beam scanning electron microscopy

A FIB-SEM is equipped with a FIB system for milling the surface of the sample. The image is taken by detecting BSE and creating TEM-like images by SEM. Serial images are acquired by repeating the process of milling and scanning the exposed surfaces (Fig. 2B). Of the three different procedures for 3D ultrastructure imaging, FIB-SEM can achieve the highest image resolution (5 nm) and the thinnest slice thickness, but the sample size is restricted to be as small as 20 μm per side due to the limited area of ion beam current. The other disadvantages of FIB-SEM are sample loss by milling, sample damage by ion beam current, and the high cost of the equipment. Ichimura et al. [10,11] and Myyaki et al. [12] conducted excellent experimental studies using FIB-SEM to demonstrate interesting ultrastructural architectures of podocytes in organ development [10] and in experiments on puromycin nephrosis in rats [11,12].

### Table 1. Comparison of three-dimensional ultrastructure analysis techniques by SEM

<table>
<thead>
<tr>
<th>Variable</th>
<th>SBF-SEM</th>
<th>FIB-SEM</th>
<th>Array tomography (ultramicrotome + SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully automated data acquisition</td>
<td>Yes</td>
<td>Yes</td>
<td>No (partly possible for cutting or imaging)</td>
</tr>
<tr>
<td>Sections preserved for post-processing and “random-access” imaging (re-imaging)</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>The maximal size of the sample (x × y)</td>
<td>500 × 500 μm²</td>
<td>20 × 20 μm²</td>
<td>3 × 3 mm²</td>
</tr>
<tr>
<td>Minimal sectioning/milling thickness</td>
<td>≥25 nm</td>
<td>≥5 nm</td>
<td>≥30 nm</td>
</tr>
<tr>
<td>Metal staining for EM</td>
<td>pre-embedding (en bloc)</td>
<td>pre-embedding (en bloc)</td>
<td>pre-embedding (en bloc) and/or post-sectioning</td>
</tr>
<tr>
<td>Immunostaining for LM</td>
<td>pre-embedding (en bloc)</td>
<td>pre-embedding (en bloc)</td>
<td>pre-embedding (en bloc) and/or post-sectioning</td>
</tr>
<tr>
<td>Problems specific to each technique</td>
<td>Slice debris, surface charging, sensitivity to electron dose</td>
<td>Redeposition of vaporized materials</td>
<td>Wrinkled sections, occasional section damage or loss</td>
</tr>
<tr>
<td>The acquisition time and dataset size for different volumes (μm³)</td>
<td>10 × 10 × 10</td>
<td>2 hours, 0.4 GB</td>
<td>39 hours, 8 GB</td>
</tr>
<tr>
<td></td>
<td>20 × 20 × 20</td>
<td>4 hours, 3.2 GB</td>
<td>10 days, 8 GB</td>
</tr>
<tr>
<td></td>
<td>50 × 50 × 50</td>
<td>22 hours, 50 GB</td>
<td>4 months, 1 TB</td>
</tr>
<tr>
<td></td>
<td>100 × 100 × 100</td>
<td>5 days, 400 GB</td>
<td>not available</td>
</tr>
</tbody>
</table>

EM, electron microscopy; FIB, focused ion beam; LM, light microscopy; SBF, serial block-face; SEM, scanning electron microscopy.

Quoted with modification in part from the article of Titze and Genoud (Biol Cell 2016;108:307–323) [4].
Array tomography

Array tomography is a method in which ultrathin sections are cut by a stationary diamond knife attached at the edge of a water-filled boat. The series of cut sections are then automatically or semiautomatically mounted on a substrate, such as a glass slide [13], silicon plate [14], or electron conductive tape [15]. Thereafter, the samples on the substrate are automatically observed by SEM connected to the sectioning equipment or are transferred to another SEM and imaged manually (Fig. 2C). The advantages of this method are its applicability to large samples of up to several millimeters (3 × 3 mm²), its preservation of the sections for subsequent electron-staining or immunostaining and reexamination, and its usefulness for CLEM [16]. Because the apparatus for cutting and mounting sections can be separated from the SEM, the cost can be reduced when the existing SEM is used for manual image acquisition. Array tomography is also a useful technology when the preserved ultrathin sections are efficiently utilized in later examinations, such as in immuno-EM, to create fascinating images of cells and tissues.

Low-vacuum scanning electron microscopy

Low-vacuum SEM (LVSEM) is designed to apply SEM to materials with poor electron conductivity. ‘Low vacuum’ means that evaporated ion particles with both positive and
negative charges are present in the SEM chamber, effectively preventing the electron charge-up phenomenon on the sample surface, the main obstacle for SEM observation, especially when it is applied to biological specimens that have not been coated with a metal. The low vacuum also allows the apparatus to be small, such as table-top size. As a result, several low-cost and easy-to-use LVSEMs have been provided by EM companies.

Inaga et al. [17,18] introduced a novel method to analyze paraffin-embedded kidney biopsy sections with LVSEM. This method enabled rapid and convenient electron microscopic observation without any special procedures for EM and provided a wide-range observation of biopsy specimens using identical sections from observation with light microscopy (LM) [19]. In this examination, periodic acid methenamine- and Masson-stained sections were useful for the observation because they contained a heavy metal, silver or tungsten, respectively, which provided metal staining for EM and led to clearer images of the glomerular structures, like GBM and mesangium (Fig. 4A). Furthermore, this method revealed the 3D glomerular morphology because the section was cut at several μm thicknesses, resulting in unexpected, interesting implications for understanding the pathology of a variety of glomerular diseases (Fig. 4B). Okada et al. used LVSEM to demonstrate 3D alterations of GBM in Alport syndrome and thin basement membrane disease [20] and podocytes and GBM in patients with pediatric nephrotic syndrome [21]. Masuda et al. [22] applied LVSEM to investigate GBM injury in IgA nephropathy and revealed GBM abnormalities combined with abnormal immunostaining of type IV collagen α2 and α5 chains in the same section of biopsy material. Recently,

Figure 4. Low-vacuum scanning electron microscopy (LVSEM) images of paraffin-embedded sections. (A) Normal rat glomerulus (×3,000) and (B) human glomerulus of membranous nephropathy (×1,500; inset, ×4,000). (A) A normal rat kidney was fixed with 2.5% glutaraldehyde and embedded in paraffin. The section was cut into 4-μm-thick slices and mounted on a glass slide. After deparaffinization, the section was stained with uranyl acetate and lead citrate by immersion. LVSEM observation was performed using a FlexSEM-1000 (30 Pa, 15 kV acceleration voltage, detected by backscattered electrons [BSE]; Hitachi Hitech, Tokyo, Japan). The three-dimensional (3D) ultrastructure of the podocyte (PD) foot process (FP) could be observed along with the cut surface of the mesangial cells (MCs) and matrix. (B) A frozen section of kidney biopsy obtained from a patient with membranous nephropathy was first used for immunofluorescent examination and then fixed with formalin, stained with periodic acid methenamine silver, and observed by LVSEM using a TM-3000 (30 Pa, 15 kV acceleration voltage, detected by BSE; Hitachi Hitech). The glomerular basement membrane (GBM) stained with silver was brightly apparent, whereas the cellular components of the glomerulus were not depicted because of a lack of silver staining. Close observation of the GBM revealed numerous small pores or craters formed in the GBM, which represented the presence of immune deposits in membranous nephropathy (white bar in inset, 2 μm). By LVSEM, 3D observation of the glomerular structures (e.g., GBM) can be easily performed using routine kidney biopsy samples in frozen or formalin-fixed paraffin-embedded sections, when heavy metal staining (e.g., silver or uranium/lead) is used.

ED, endothelial cell; PEC, parietal epithelial cell; RBC, red blood cell.

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two studies on kidney allografts demonstrated that LVSEM was useful for early diagnosis for transplant glomerulopathy in chronic antibody-mediated rejection [23,24].

Recently, Sawaguchi et al. [25] reported a precise method to perform an informative 3D survey using a 30-μm-thick paraffin section, facilitating the investigation of face-side (instead of sectioned) images of cell/tissue architectures, rarely seen in the usual thin-section observations. In research practice, Mukai et al. [26] established a novel and convenient method to demonstrate the 3D structure of endothelial glycocalyx observed by LVSEM.

We anticipate that LVSEM can give useful information in the ordinary diagnosis of kidney biopsy, providing insights into the glomerular pathology. In 2017, the study organization of LVSEM for Renal Biopsy was founded by Japanese renal pathologists and nephrologists interested in and engaging in this enterprising technique (https://lvsem.org). We hope that the activity of this study organization will contribute to the progress in the research and clinical practice of nephrology in the future.

Correlative light and electron microscopy

Correlative light and EM (CLEM) is a method to correlate an LM image with an EM image, enabling visualization of the ultrastructural morphology of the region of interest where the LM marker, such as a fluorescent protein or dye, is located [27,28]. Because CLEM can be applied to several methodologies, including different microscopic modalities and image processing techniques, it has great potential for use in future research in a variety of fields of medicine and science. The LM image is usually taken from the section using immunofluorescence (IF) or immunohistochemistry (IHC), and the EM image is taken from the same section after metal staining for TEM or SEM. Then, both images are merged by a digital graphic application. The following procedures are performed in a typical CLEM analysis: 1) IF or IHC staining, 2) LM imaging, 3) heavy metal coating (sputtering) or staining, 4) EM imaging by TEM or SEM, and 5) merging both images by graphic software. At the beginning of CLEM, Takizawa and Robinson [29] introduced a method to stain the ultrathin section cut from a resin-embedded block using a bifunctional marker for IF

Figure 5. Correlative light and electron microscopy (CLEM) images of neutrophil extracellular traps (NETs) and fibrin fibrils of a lung from a human autopsy. (A) Lung tissues from an autopsy of legionnaire’s pneumonia, fixed with formalin and embedded in paraffin, were examined with immunofluorescent imaging to reveal fibrinogen gamma chain (FGG) in red, lactoferrin (LF) in green, and nucleic acid (DAPI) in blue, which were observed by confocal laser scanning microscopy (CLSM, LSM710; Carl Zeiss, Oberkochen, Germany). (B) The same region of the same section was observed and imaged by scanning electron microscopy (SEM, S-4000; Hitachi Hitech, Tokyo, Japan). (C) Both images were merged. NETs are observed as a cluster of globular materials containing LF (blue) and nuclear materials containing DAPI (blue). Simultaneously, thick fibrous bundles are formed as a cluster of fine fibrin filaments containing FGG (red). Thus, the CLEM image demonstrated the ultrastructural changes of NETs and fibrin where fibrin filaments were attached and intermingled with globular materials in the legionnaire’s pneumonia lung of the autopsied patient. Scale bars = 5 μm. Reprinted from the article of Onouchi et al. (Acta Histochem Cytochem 2016;49:141–147) [31] with original copyright holder’s permission.
Figure 6. Schematic illustration of the detection system in scanning transmission electron microscopy (STEM). In STEM, an emitted electron beam permeates through the sample and hits a reflection plate located under the sample. After that, the signal is generated as secondary electrons (SE) and is detected by an ultra variable detector (UVD) with scanning electron microscopy. Some SE are generated from the sample surface and also detected by the UVD. Because the sample is thicker, noise signals are easily generated from the sample and the reflection plate, increasing the signal-to-noise ratio and interfering with clear imaging. Therefore, a novel scintillation holder is installed under the sample, which enables an increase in the STEM signal and improves the detection of UVD.

and EM, a fluorescent ultrasmall immunogold probe, FluoroNanogold (Nonoprobes, Yaphank, NY, USA), and then observed the stained section by TEM. Later, Koga et al. [30] developed another CLEM method using semi-thin resin sections stained by fluorescent nanogold and observed the same fields by CLSM and SEM. Recently, SEM has been preferred for EM imaging, resulting in a variety of samples for immunostaining methods. Onouchi et al. [31] performed an excellent CLEM study that employed CLSM and SEM using formalin-fixed paraffin-embedded autopsy lung sections to demonstrate high-resolution 3D images of neutrophil extracellular traps (NETs) and fibrin fibrils (Fig. 5). These technologies can be applied to kidney biopsy samples, but several critical conditions must be investigated carefully, such as fixation, embedding, and sectioning, to preserve the morphological quality for ultrastructural observation.

Scanning transmission electron microscopy

A higher resolution can be achieved by the stronger acceleration voltage in TEM and a more focused electron beam in SEM. Scanning transmission EM (STEM) is a method to combine both procedures to greatly improve the resolution. This method observes ultrathin sections not by the broad electron beam used in TEM but by the focused scanning electron beam used in SEM and has been employed in material science and technology, achieving a resolution of 40.5 pm [32]. However, STEM has not been applied to biological specimens because it is unsuitable for low-contrast specimens. Aoyama et al. [33] have applied STEM to observe cultured cells embedded in resin and cut into 1-μm-thick sections. In their study, STEM tomography successfully demonstrated very fine ultrastructural 3D images of mitochondria. In STEM, the emitted electron beam permeates through the sample and hits the reflection plate located under the sample. After that, the signal is generated as SE and detected by an ultra variable detector (UVD) in the SEM (Fig. 6). Because the sample is thicker, noise signals are easily generated from the sample and the reflection plate, increasing the signal-to-noise ratio and interfering with clear imaging. A novel scintillation holder is installed under the sample instead of the reflection plate to overcome this problem, enabling an increase in STEM signal and improving the detection by the UVD [34]. Using STEM methods, we can demonstrate a high-resolution TEM-like image by observing the ultrathin section of human kidney biopsy samples with SEM instead of TEM (Fig. 7). The technical advances in STEM will soon promote applying STEM to biological research and provide plenty of insights to understand the pathology of glomerular disease. It may also dramatically improve the resolution quality of LVSEM, allowing LVSEM to serve as a substitute for TEM in future clinical practices of kidney biopsy diagnosis.

Conclusions and future prospective

TEM has been necessary for the diagnosis of kidney biopsy, and this situation will not change. However, the technical complexity and high cost of TEM limit its use in many hospitals and institutes. Recent advances in EM, especially in SEM, promote the expansion of the use of SEM in a variety of directions, such as TEM-like imaging by SEM without...
Figure 7. Scanning transmission electron microscopy (STEM) images of kidney biopsy samples obtained from a patient with lupus nephritis (A, B; ×8,000) and amyloidosis (C, D; ×50,000). The original STEM image (A, C) detected by backscattered electrons (BSE) is the opposite of the transmission electron microscopy (TEM) image in black-and-white color. Therefore, a color conversion is required to obtain a TEM-like image (B, D). STEM technology enables the performance of high-resolution analysis comparable to TEM by using SEM installed with a novel scintillation holder to reduce noise for clearer imaging. The images were taken by a SU-5000 (30 kV acceleration voltage; Hitachi Hitech, Tokyo, Japan) and detected by BSE.

complex techniques, automated 3D ultrastructural imaging, application of paraffin-embedded sections for ultrastructural observation by LVSEM, and STEM techniques to improve the resolution of ordinary SEM. In addition, CLEM imaging can provide fantastic images to help understand the physiology and pathology of various organs and cells. EM technology has progressed in this half-century, and many advances in renal pathology and nephrology are indebted to EM. We believe this situation will be the same during the next half of the century and provide plenty of novel insights for understanding the pathogenesis and diagnosis of a variety of glomerular diseases in the future.

Conflicts of interest

All authors have no conflicts of interest to declare.

Authors’ contributions

Conceptualization: KH, TT
Data curation: KH, TT, DK
Writing–original draft: KH, TT
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References


Histologic evaluation of activity and chronicity of lupus nephritis and its clinical significance

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The National Institutes of Health (NIH) lupus nephritis activity and chronicity indices, which comprise six activity scores and four chronicity scores, have a long development history. The 2018 revised International Society of Nephrology/Renal Pathology Society classification for lupus nephritis adopted the most recent NIH indices to replace subclasses A, C, and A/C. Although an evidence-based approach should further evaluate the clinical significance of the modified NIH indices, recent validation studies demonstrated that the modified chronicity indices have a strong correlation with kidney outcome of lupus nephritis.

**Keywords:** Biopsy, Lupus nephritis, Prognosis, Treatment outcome

**Introduction**

Lupus nephritis is a renal manifestation of systemic lupus erythematosus (SLE) that displays a broad spectrum of histologic changes. The International Society of Nephrology/Renal Pathology Society (ISN/RPS) lupus nephritis classification is the most widely used system for categorizing glomerular lesions of lupus nephritis according to the location of immune complex deposition \(^1,\)\(^2\). Since its creation in 2004, improvements in the classification system have been discussed \(^3\), and proposed revisions were published in 2018 \(^4\), such as phase 1 and phase 2 recommendations, which were based on both already published evidence and those lesions needing further studies, respectively. The phase 1 recommendations include refined definitions and newly adopted or refined terminology, such as lesions of mesangial hypercellularity, endocapillary hypercellularity, fibrous or fibrocellular crescents, adhesion, and fibrinoid necrosis. The elimination of the subclasses for class IV (class IV-S and class IV-G) was also proposed because of
the uncertain clinical significance and unclear parameters for distinguishing them, which could result in interobserver variability. Regarding disease activity, the authors recommended that the original ISN/RPS designations of activity and chronicity using subclasses A, C, and A/C should be replaced by the modified National Institutes of Health (NIH) lupus nephritis activity and chronicity scoring system (NIH indices) that originated in the previous World Health Organization classification.

This review focuses on pathological evaluation of lupus nephritis disease activity including the history of these NIH indices and their clinical significance, the recent revision of the ISN/RPS classification, and the results of validation studies on the classification’s use. Other approaches to disease activity assessment using histologic evaluation will also be discussed.

**Development of the National Institutes of Health indices**

Concepts regarding active and chronic lesions in lupus-related kidney lesions and their inclusion into lists can be found in the literature as early as 1979 in an NIH conference report on SLE [5]. In this report, parameters for the activity index (AI) included four glomerular lesions and one tubulointerstitial lesion, and the parameters for the chronicity index (CI) included two glomerular lesions and two tubulointerstitial lesions. Each parameter was scored from 0 to 3+. The original NIH indices, which are similar to current NIH indices, were proposed in research articles by Austin et al. in 1983 [6] and 1984 [7]. In pursuit of histologic predictors of kidney failure in cases of lesions then classified as “diffuse proliferative” or “membranoproliferative” lupus nephritis, the authors adopted these previously defined parameters [5] and developed a semiquantitative scoring system for both active and chronic lesions. Of note, in older studies, the term focal or diffuse “proliferative” lupus nephritis was often used. It is now recognized that these hypercellular lesions include mostly infiltrating inflammatory cells, and thus the term “endocapillary hypercellularity” is now used to describe these active lesions seen in class III or class IV lupus nephritis. Parameters listed for active lesions included glomerular cell proliferation, which corresponds to endocapillary hypercellularity, leukocyte exudation, karyorrhexis/fibrinoid necrosis, presence of cellular crescents, extent of so-called “hyaline” deposits, and degree of interstitial inflammation (Fig. 1). Of note, the term “hyaline” deposits or “hyaline thrombi” was used to describe large, glassy eosinophilic, usually subendothelial, deposits seen by light microscopy, protruding into the capillary lumen. This term is misleading because the deposits are not truly within capillary lumina and do not consist of fibrin, and thus are not true thrombi. All parameters were scored from 0 to 3+, according to the percentage of affected glomeruli (<25%, 1+; 25%–50%, 2+; and >50%, 3+), for an evaluation of glomerular cell proliferation, karyorrhexis/fibrinoid necrosis, and presence of cellular crescents. Scoring according to degree—mild/few (1+), moderate (2+), and severe/extensive (3+)—was applied to the evaluation of leukocyte exudation, hyaline deposits, and interstitial inflammation [7]. Glomerular sclerosis (both global and segmental), presence of fibrous crescents, tubular atrophy, and interstitial fibrosis were listed as parameters of chronic lesions and scored in a similar way to those of active lesions; the percentage of affected glomeruli (for glomerular sclerosis and fibrous crescents) or mild, moderate, and severe changes (for tubular atrophy and interstitial fibrosis) [7]. The karyorrhexis/fibrinoid necrosis and cellular crescents scores were weighted two-fold because these lesions were listed as particularly active parameters in previous studies [8,9]. Therefore, the maximum scores were 24 for active lesions (AI) and 12 for chronic lesions (CI) [7]. This scoring system has been adopted by other studies of histologic evaluation of lupus nephritis [10–14] and is currently included in most major renal pathology textbooks [15–18].

**Clinical significance of the National Institutes of Health indices**

In the original report by Austin et al. [6], the NIH indices had a predictive value for identifying patients at high risk of kidney failure. Patients with an AI of 11 or more and a CI of 3 or more constituted the high-risk group [6]. When focusing on cases of diffuse proliferative lupus nephritis, AI, CI, and individual components of the CI had predictive value, with high-risk patients scoring 12 or more points in the AI and 4 or more points in the CI [7]. The predictive values of both the AI and CI were consistent in patients with severe lupus nephritis who had been treated with either cyclophosphamide or methylprednisolone [19].
We will now review a number of studies assessing the predictive powers of AI and CI. The studies are arranged according to the similarity of conclusions and not chronologically. Magil et al. [10] used the same parameters and scoring methods as reported by Austin et al. [6] in a study of diffuse proliferative lupus nephritis and reported that AI, as well as other clinical and histologic features, predicted outcomes. Interestingly, weighting the scores of cellular crescents and fibrinoid necrosis/karyorrhexis did not affect the significance of the AI [10]. Esdaile et al. [11–14] also adopted the NIH indices in their studies and demonstrated the predictive values of both the AI and CI in renal outcomes. Arce-Salinas et al. [20] reported that patients with diffuse proliferative lupus nephritis with an AI score higher than 9 had significantly higher risk of developing chronic renal failure than patients with an AI score less than or equal to 9; Yokoyama et al. [21] reported that AI was the most significant risk factor for death and/or end-stage kidney disease after initial kidney biopsies. However, Appel et al. [22] reported that the AI and CI did not significantly predict kidney outcomes, and a substantial proportion of mesangial lesions accounted for these discrepancies. Schwartz

**Figure 1. Example lesions included in the National Institutes of Health indices (×400).** (A) Endocapillary hypercellularity (arrow; hematoxylin and eosin stain). (B) Cellular crescent (periodic acid-Schiff stain). (C) Fibrinoid necrosis (arrow; periodic acid-methenamine silver stain). (D) Hyaline deposits (wire-loop lesions, arrows; acid fuchsin orange G stain).
et al. [23] also reported that AI and CI did not significantly predict kidney failure or death in patients treated for severe lupus nephritis. Levey et al. [24] conducted a multicenter, randomized controlled study and did not find significant differences in AI and CI between groups of patients with and without kidney failure who had been treated for severe lupus nephritis. Wu et al. [25] found that both the AI and CI were significant predictive factors for kidney outcomes in a univariate Cox hazard analysis, and the CI remained significantly predictive in multivariate analysis. Hsieh et al. [26] performed a cohort study and showed that patients with a CI greater than 3 were more likely to progress to kidney failure, whereas the AI was not predictive of kidney survival.

However, Schwartz et al. [27] also demonstrated the irreproducibility of the AI and CI among four pathologists, showing a reliability coefficient of 0.48 for AI and 0.57 for the CI. These authors attributed the low reliability coefficient to interpretative differences. Dasari et al. [28] analyzed pathologist concordance using the ISN/RPS classification and the AI and CI of patients with lupus nephritis after reviewing six studies with at least four pathologists involved in each study. These authors concluded that the AI and CI exhibit poor interobserver agreement and are therefore limited for clinical use.

Since its inception, studies assessing the prognostic importance of the AI have concluded it has low utility, and most data collected over time indicate that the AI is not associated with kidney outcome [22–26]. The reason for this shift in utility may be due to responsiveness of certain active lesions to immunosuppressants. For example, patients with high AI scores may be more likely to receive more aggressive immunosuppressive therapy, and when these therapies are effective, AI score would not be associated with worse outcome. The Oxford study on immunoglobulin A (IgA) nephropathy previously suggested that endocapillary hypercellularity is a lesion that is more responsive to immunosuppressants due to a lack of association with kidney failure in patients who received immunosuppression [29]. Further studies showed that crescents in IgA nephropathy were not associated with worse outcome if present in <25% of glomeruli when the patient was treated with immunosuppression [30]. Similarly, some researchers suggested that the endocapillary hypercellularity of lupus nephritis may be reversible in patients using immunosuppressants, given the lack of the lesion’s association with a decline in kidney function [31,32].

**Revision of the National Institutes of Health indices and their incorporation into the International Society of Nephrology/Renal Pathology Society classification**

The original ISN/RPS classification referred to the NIH indices as a possible supplement for the A, C, and A/C subclasses of lupus nephritis classes III and IV [1,2] because these subclasses do not describe the extent of active and chronic lesions [3]. In the 2018 proposed revision, the NIH indices were modified, and it was suggested they be incorporated into the ISN/RPS classification, even replacing the A, C, and A/C subclasses. The proposed modification included separating karyorrhexis from fibrinoid necrosis and combining it with neutrophil infiltration (leukocyte exudation in the original NIH indices) and including fibrocellular crescents as well as cellular crescents in AI and to provide clearer definitions of these lesions (Table 1) [4]. Importantly, this incorporation does not mean that the scientific basis of the NIH indices has been confirmed. The NIH indices were not originally established using an evidence-based approach, and clinical validation of these indices has not been thoroughly investigated, as mentioned above. There-

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<table>
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<tr>
<th>Index</th>
<th>Score^a</th>
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<tr>
<td>Modified activity indices</td>
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<tr>
<td>Endocapillary hypercellularity</td>
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<td>Neutrophils/karyorrhexis</td>
<td>0–3</td>
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<td>Fibrinoid necrosis</td>
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<td>Hyaline deposits</td>
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<td>Cellular/fibrocellular crescents</td>
<td>(0–3) × 2</td>
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<td>Interstitial inflammation</td>
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<td>Modified chronicity indices</td>
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<tr>
<td>Total glomerulosclerosis^b score</td>
<td>0–3</td>
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<tr>
<td>Fibrous crescents</td>
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<td>Tubular atrophy</td>
<td>0–3</td>
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<td>Interstitial fibrosis</td>
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^a 1; <25%; 2, 25%–50%; 3, >50% glomeruli/tubules/cortical interstitium involved. ^b Global and/or segmental sclerosis.
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Modified from the article of Bajema et al. (Kidney Int 2018;93:789-796) [4] according to the Elsevier user license.
fore, the authors of the proposed revision of the ISN/RPS classification proposed that the validity of the NIH indices should be confirmed using an evidence-based approach in a phase 2 modification of classification of kidney lesions in SLE [4].

**Validation studies of the modified National Institutes of Health indices**

Phase 2 analysis to assess the validity of the NIH indices has not yet been done; however, there have been several validation studies of the modified NIH indices. Tao et al. [33] performed a retrospective validation study of the revised ISN/RPS classification system in a Chinese cohort at a single institution. They evaluated the diagnostic reproducibility of each histologic parameter of the revised ISN/RPS classification, the correlation of histologic parameters and clinical/laboratory features at the time of biopsy, and the correlation between histologic parameters and long-term outcomes. The authors observed that the modified CI, compared with the original CI, had a better correlation with composite outcomes, including death, progression to end-stage kidney disease, and a 30% reduction in the estimated glomerular filtration rate (eGFR). The original CI did not correlate with outcomes. The modified AI did not show significant differences in correlations versus the original AI. The authors also showed that the diagnostic reproducibility of assessment of cellular crescents was poor but was markedly increased when cellular and fibrocellular crescents were considered together [33]. We think it is possible that the clear distinction between the types of crescents was the reason for the increased correlation of the modified CI with outcomes. Umeda et al. [34] also performed a retrospective comparison of the original and revised ISN/RPS classifications, including the NIH indices, at a single Japanese institution. They compared the modified NIH indices with the A, C, and A/C subclasses of the original ISN/RPS classification in terms of their correlation with a 30% decline in eGFR. Their analysis indicated that the revised ISN/RPS classification and the modified NIH indices were superior to the original versions. The modified CI (but not the modified AI) correlated with kidney outcomes, while subclass type did not. The incorporation of the modified NIH indices into the revised ISN/RPS classification was evidence of progress, as the authors discussed, considering that tubulo-interstitial lesions were included in the classification and that modified NIH indices provided information on the quantity of histologic parameters, even in cases of lupus nephritis classes I, II, and V. It is also worth noting that the authors used cut-off scores of 8 (≤8 vs. ≥9) for the modified AI and 4 (≤4 vs. ≥5) for the modified CI to compare kidney outcomes. Another retrospective validation study focusing on the clinical usefulness of the modified NIH indices was conducted by Nakagawa et al. [35] in a cohort at a single Japanese center. The authors grouped patients with low, moderate, and high AI and CI scores (low: 0–5, moderate: 6–11, and high: 12–24 for the AI; and low: 0–2, moderate: 3–5, and high: 6–12 for the CI). Using a multivariable analysis and adjusting for age and serum creatinine levels, moderate and high modified CI scores were significantly correlated with composite endpoints of end-stage kidney disease or all-cause death. The modified AI was not a risk factor for outcomes in multivariable analysis. A study from Thailand demonstrated that revised CI was one of the most significant predictors of clinical remission of so-called “proliferative” lupus nephritis after induction therapy, although the study was performed in a small group of patients [36]. Navarro et al. [37] analyzed the predictability of Austin’s morphological indices in a study at a single Portuguese center. Although the definition of indices differed slightly from that of the modified NIH indices, increased CI still correlated with worse kidney function and proteinuria at the end of follow-up. AI correlated with laboratory parameters reflective of immunological activity (C3, C4, and anti–double-stranded DNA) at presentation. Another retrospective multicenter cohort study comparing the predictability of the A/C subclass and the modified NIH indices (AI and CI) was performed by Hachiya et al. [38]. In this study, a higher CI score (not A/C subclass or AI) was associated with both decline in kidney function (as defined by a 1.5-fold increase in serum creatinine level) and complete remission by Cox regression analysis.

**Histologic indicators of disease activity other than National Institutes of Health indices**

There have been attempts to invent a morphologic index that is more closely correlated with a patient’s prognosis and predictive of treatment response than the NIH indices. Of those, one is the well-known Biopsy Index proposed by
Hill et al. [39]. By modifying the standard AI and CI with the addition of new indices encompassing tubulointerstitial lesions and immunofluorescent findings, Hill et al. [39] developed this index, which is a combination of four biopsy indices: the Glomerular Activity Index, the Tubulointerstitial Activity Index, the Chronic Lesions Index, and the Immunofluorescence Index. Conclusive analyses and comparisons with other histologic indices have revealed that the Biopsy Index is more significantly associated with parameters of clinical and kidney survival, both for the initial biopsy and for patients with a second protocol biopsy that was performed after 6 months of induction therapy. However, Rathi et al. [40] found no significant correlation between the Biopsy Index and parameters of clinical outcomes; however, the sample size was small and retrospectively analyzed.

### Conclusion

Since they were proposed by Austin, there have been many studies on the clinical significance of the NIH indices. At first, it seemed that both the AI and CI were correlated with kidney outcomes. Over time, the CI proved to be more strongly predictive of kidney outcomes than the AI, possibly reflecting changes in therapeutic strategies for treatment of active lesions. The A/C subclass was proposed to be substituted by the modified NIH indices in the 2018 ISN/RPS revised classification of lupus nephritis with the caveat that an evidence-based approach should be followed for validation. Several validation studies have demonstrated a stronger correlation of the modified CI to kidney outcome than either the original NIH AI or CI. Given the less predictive power of the modified AI, the modified CI is expected to be recognized as the primary parameter for kidney outcomes of lupus nephritis. The next revision of the lupus nephritis classification should further clarify this issue.

### Conflicts of interest

The authors have no conflicts of interest to declare.

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Introduction

Due to the increased risk of end-stage renal disease and its devastating effects on the cardiovascular system, chronic kidney disease (CKD) is associated with high morbidity and mortality. There is a growing global burden of CKD, affecting 10% of adults worldwide; meanwhile, the global mortality rate attributed to CKD has increased by 41.5% in the last three decades \(^1,2\). Tubulointerstitial fibrosis, a defining feature of CKD, is characterized by extracellular matrix (ECM) accumulation and renal scarring, which lead to both structural and functional deterioration of the kidneys. The fibrogenesis process includes inflammatory cell infiltration, excessive fibroblast activation, overwhelming ECM deposition, tubular atrophy, and renal microvascular rarefaction. Recent advances in single-cell RNA sequencing (scRNA-seq) have enabled tremendous progress in understanding the mechanisms behind renal fibrosis.

In past decades, clinically available pharmacological interventions for delaying CKD progression have been primarily restricted to renin-angiotensin-aldosterone system inhibitors. Transforming growth factor \(\beta\) (TGF-\(\beta\)) is the master regulator of fibrosis, and new agents that target the TGF-\(\beta\) signaling pathway are continually emerging. Particularly, there is mounting evidence supporting the critical role of extracellular vesicles (EVs) in renal physiology and pathology. EVs are considered key mediators of cellular communication participating in renal fibrosis progression.

Keywords: Chronic kidney disease, Extracellular vesicles, Mechanism, Renal fibrosis, Therapy
Importantly, EVs are promising therapeutic vectors due to their intrinsic contents and natural nanocarrier properties for small-molecule drugs as well as genetic therapies.

The purpose of this review is to provide new insights into the mechanisms of renal fibrosis, as well as prospective therapeutic approaches targeting pathological signaling and cellular events. The important role of EVs will be emphasized regarding the mechanisms and therapy of renal fibrosis.

**Pathogenesis of renal fibrosis**

An overview of the complex interplays and critical events involved in renal fibrosis progression is shown in Fig. 1.

**Figure 1. Schematic elucidation of cellular and signaling events in renal fibrosis.** Renal tubule injury acts as a driving force in fibrosis progression through communication with immune cells, peritubular capillary (PTC), and interstitial stroma cells via soluble or extracellular vesicle (EV) signaling. Persistent or severe injury leads to maladaptive repair of tubular epithelial cells (TECs) and subsequent EMT or pEMT, contributing to renal fibrosis. PTC rarefaction generates a hypoxic environment that promotes tubular atrophy. The phenotypic heterogeneity and functional plasticity elucidate the versatile roles of macrophages during inflammation, tissue repair, and fibrosis. Excessive accumulation of ECM components contributes to overactivation of myofibroblasts originating from multiple cellular sources and provides a substrate for latent transforming growth factor β (TGF-β) activation. Endogenous EVs play a notable role in delivery of messages in cellular communication, while exogenous EVs are being developed as new therapeutic agents for renal fibrosis.

AKI, acute kidney injury; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; EndoMT, endothelial-mesenchymal transition; MSC, mesenchymal stem cell; pEMT, partial epithelial-mesenchymal transition.
macrophages, induce further TEC injury and necrosis [5,6]. Unresolved or excessive tubulointerstitial inflammation can lead to persistent kidney injury, which plays a central role in maladaptive repair of TECs.

A process of epithelial-mesenchymal transition (EMT) after injury has long been recognized and contributes to renal fibrosis as epithelial cells switch to mesenchymal cells [7]. However, whether epithelial cells undergo complete EMT and become matrix-producing cells depends on the condition of tissues and persistence of cytokine production [7]. Nevertheless, it has been demonstrated that de-differentiated TECs remained adherent to the membrane and express markers of both epithelial and mesenchymal cells, a phenomenon called partial EMT (pEMT), contributing to renal fibrogenesis [8]. Importantly, injured and de-differentiated proximal tubular cells are responsible for tissue repair other than fixed tubular progenitor cells, and proliferation of proximal tubules might be regulated by the EGFR-FOXM1 signaling pathway [9].

Recently, several scRNA-seq analyses further verified that maladaptive repair of TECs accelerates renal fibrosis. The scRNA-seq of a mouse AKI model identified a distinct proinflammatory and profibrotic role of failed-repair proximal tubule cells [10]. Another study found that maladaptive repair of proximal tubules could accelerate progressive interstitial fibrosis, which consequently promotes pericyte activation, peritubular capillary (PTC) loss, and matrix deposition [4]. In addition, new clusters of proximal tubular cells (present only following injury) with the ability to transfer pathological signaling to fibroblasts and macrophages were identified [11]. However, the underlying cellular and molecular mechanisms of maladaptive repair remain to be fully elucidated.

Peritubular capillary rarefaction

PTC rarefaction along with tubular atrophy is commonly detected in renal fibrosis. The level of PTC loss correlates with the severity of fibrosis [12]. Animal experiments have confirmed in CKD models such as the remnant kidney model [13] and unilateral ureteral obstruction (UUO) [14] that capillary density was negatively correlated with fibrosis. An antiangiogenic environment including deprivation of endothelial cell survival factors, upregulation of anti-angiogenic factors, dysfunction of endothelial cells, and loss of endothelial cell integrity contributes to the rarefaction of PTC [15]. In addition, pericyte disintegration and loss after kidney injury promoted instability of blood vessel structure and further capillary rarefaction. To date, the mechanism of PTC rarefaction is not clearly identified. However, inflammatory macrophages can block expression of tubular vascular endothelial growth factor A by infiltration and secretion of inflammatory cytokines, especially interleukin (IL) 1β and tumor necrosis factor α [16]. This blockage is regarded as a core event for PTC rarefaction. Additionally, as a key feature in ischemic kidney injury, the endothelial-to-mesenchymal transition (EndoMT) is depicted as the transition from typical endothelial cells to a profibrotic phenotype [17], which results in PTC rarefaction and CKD progression.

The versatile roles of macrophages

The phenotypic heterogeneity and functional plasticity elucidate the versatile roles of macrophages during tissue repair and fibrosis. Lineage tracing studies indicate that self-renewed kidney resident macrophages (KRMs) in adult kidneys largely originate from yolk sac erythro-myeloid progenitors (EMPs), fetal liver EMPs, and hematopoietic stem cells [18]. Once injury occurs, circulating monocytes from bone marrow infiltrate the kidney in inflammatory microenvironments [19]. A recent study demonstrated that KRMs and monocyte-derived infiltrated macrophages (IMs) play complementary functions in reducing tissue inflammation and fostering tissue repair [20].

Traditionally, IMs in kidney disease are grouped into either classically activated M1 macrophages associated with the TH1-like response or alternatively activated M2 macrophages that contribute to the TH2-like response. Specifically, M2 macrophages can be subdivided into three types based on diverse stimuli and functions [21]. Accumulation and activation of macrophages are directly related to kidney injury and fibrosis severity, and excessive profibrotic mediators secreted from M2 macrophages could drive myofibroblast proliferation and profibrotic signaling pathways [22]. Recent studies have illustrated that Ly6C<sup>high</sup> monocytes accumulate in the inflammatory kidney and differentiate into three subpopulations, including the proinflammatory CD11b<sup>+</sup>/Ly6C<sup>high</sup> population presented at the onset of renal injury, the CD11b<sup>+</sup>/Ly6C<sup>int</sup> population dominant in
the renal repair phase, and the profibrotic CD11b+/Ly6C\textsuperscript{lo} population that emerges in renal fibrosis [23].

In scRNA-seq analysis, macrophage diversity can be deciphered unbiasedly or macrophage clusters can be explored according to phenotype and cell function, showing the complexity of macrophages [24]. For example, recent scRNA-seq research identified a unique population of S100A9\textsuperscript{hi}Ly6C\textsuperscript{hi} IMs mediating the initiation and amplification of inflammation in AKI, and blockade of S100a8/a9 signaling exhibited renal protective effects in an ischemia-reperfusion injury (IRI) model [20]. Therefore, a precise understanding of the dynamics and functional characteristics of macrophages under different microenvironments could offer specific therapeutic targets for kidney diseases.

**Activation of matrix-producing cells**

Excessive ECM accumulation is the key characteristic of renal fibrosis, and studies have been conducted to define the cellular sources contributing to pathological deposition of ECM. Myofibroblasts are commonly regarded as the predominant matrix-producing cell in diseased kidneys [25]. Renal resident fibroblasts can transdifferentiate into myofibroblasts with reduced production of fibroblast-derived erythropoietin, leading to renal anemia and consequent CKD progression [26].

Traditionally, α-smooth muscle actin is considered the marker for myofibroblasts, while a recent scRNA-seq study proposed Postn as another identifier for myofibroblasts with high ECM production [27]. Interestingly, there is high heterogeneity of myofibroblasts in terms of cell origin and function, similar to diverse macrophages in diseased kidneys. scRNA-seq applied in human kidney fibrosis revealed that myofibroblasts mainly originate from diverse resident mesenchymal cells, primarily distinct fibroblast and pericyte populations, far more than from fibrocytes [27]. Mesenchymal stem cells (MSCs) were previously proposed as contributors of myofibroblasts [28], and new evidence suggested that Gli1\textsuperscript{+} MSC-like cells represent a myofibroblast pool in response to injury and contribute to fibrosis development [29,30]. Although EMT and EndoMT are mechanisms involved in renal fibrosis, myofibroblasts from transdifferentiated renal tubular cells or endothelial cells have been reported to account for only a small fraction [27].

Overall, myofibroblasts are responsible for excessive ECM synthesis and deposition, and further research is needed to clarify the full map of matrix-producing cells during fibrosis development.

Myofibroblasts produce collagen fibers when activated, resulting in excessive ECM deposition. The remodeling of ECM is in an equilibrium process. The ongoing ECM protein synthesis and degradation are orchestrated by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), both of which are considered key enzymes responsible for remodeling of ECM. Thus, dysregulation of MMP/TIMP activity is associated with progression of renal fibrosis [31]. Interestingly, the mechanical structure of ECM is not simply a scaffold, but rather a substrate to bind growth factors, particularly latent TGF-β1. ECM is capable of activating TGF-β1 by supplying the necessary mechanical resistance. The latent TGF-β-binding protein (LTBP) covalently binds the latency-associated peptide (LAP) together with TGF-β to form the large latent complex. LTBP interacts with ECM components and localizes latent TGF-β in the ECM. When integrins on the cell surface attach to the Arg-Gly-Asp (RGD) binding site of LAP, TGF-β1 is released and activated through tension generated between integrins and ECM [32]. Moreover, activation of latent TGF-β is enhanced as ECM stiffness increases, leading to increased TGF-β1 signaling [33,34].

**Inflammation and fibrosis signaling activated by extracellular vesicles**

EVs are small membrane vesicles of two major subtypes: exosomes (40 to 160 nm), which originate from endosomes, and ectosomes (50 nm to 1 μm), which are derived from direct plasma membrane budding [35]. Increasing evidence supports the idea that EVs selectively transfer specific signals to regulate organ development, immune responses, and disease. Therefore, understanding the signals transferred by EVs may help shed light on the mechanisms of renal fibrosis.

As the primary component of the tubulointerstitium, TECs are particularly vulnerable to injury, which accelerates renal disease progression. The secreted proinflammatory mediators then guide inflammatory cells, including monocytes/macrophages, dendritic cells, neutrophils, lymphocytes, and mast cells, to the injured sites to provoke
inflammation and cell death [6,36]. Recent studies support a notable role of EVs in renal inflammation via mediation of tubular-macrophage crosstalk. After injury, TECs increase the secretion of EVs carrying proinflammatory-related cargoes, such as CC-chemokine ligand 2 (CCL2) messenger RNA (mRNA) and functional microRNA (miRNAs: miRNA-23a, miRNA-19b-3p, etc.), which are transferred to initiate macrophage activation and migration and augment tubulointerstitial inflammation [37-39]. In addition, EVs are essential signal messengers in the proximal-to-distal tubular communication in pathological conditions [40]. Moreover, EVs could also participate in renal fibrosis via communication with interstitial fibroblasts. Injured TEC-derived exosomes enriched with TGF-β1 mRNA promote fibroblast activation [41]. Furthermore, increasing reports suggest that EVs containing various miRNAs (miR-196b-5p, miR-150, and miR-21) can activate fibroblasts and intensify renal fibrosis [42-44]. Recently, tubular cell-derived exosomal osteopontin was identified as responsible for activation of fibroblasts and promotion of renal fibrosis development [45].

Therefore, EVs released from injured renal cells are loaded with signal molecules of inflammation and fibrosis, which favor amplification of unresolved and prolonged inflammatory proteins and further serve as a crucial trigger of tissue fibrogenesis.

**Therapy of renal fibrosis**

**Emerging transforming growth factor-β–targeted treatment**

Fibrosis is the ultimate common pathway for CKD in spite of the underlying etiology, and antifibrotic agents are crucial for treatment of CKD. Here, we mainly discuss emerging therapeutic options targeting TGF-β in renal fibrosis and CKD.

TGF-β is linked with fibrosis of various organs. Previous evidence demonstrated that TGF-β participates in pathological fibrosis processes, including mediating ECM dysregulation, transdifferentiation of intrinsic cells, and mesangial cell proliferation. Therefore, TGF-β signaling represents a critical target for renal fibrosis.

Pirfenidone is a small synthetic inhibitor that blocks the TGF-β promotor and has antifibrotic and anti-inflammatory properties. It has been widely used for idiopathic pulmonary fibrosis treatment in clinical studies [46]. In many animal models of renal disease, pirfenidone also exerts similar effects [47], however, its potential in clinical settings remains to be investigated. The ongoing TOP-CKD trial (NCT04258397), the largest pirfenidone phase II study enrolling 200 participants, is estimated to be completed by December 2024. Similarly, pentoxifylline, a clinically available drug, was reported to downregulate TGF-β1 expression, delay progression of CKD, and reduce cardiovascular risk [48].

Compared to TGF-β deficiency probably causing severe immune dysregulation, antibody neutralization of TGF-β is recognized to have higher security with fewer adverse effects. Multiple preclinical investigations have revealed that direct TGF-β neutralization could halt the development of fibrosis. Fresolimumab and LY2382770, human monoclonal antibodies that neutralize TGF-β1, have been evaluated in phase II trials in patients with steroid-resistant focal segmental glomerulosclerosis (FSGS) and diabetic nephropathy, respectively [49,50]. Unfortunately, neither achieved the expected clinical outcome, which may need to be confirmed by larger and more robust studies. Since integrin αvβ6 can activate latent TGF-β, targeted integrin αvβ6 blockade by antibodies or small molecules offers an option for inhibition of TGF-β-induced fibrosis. The monoclonal antibody STX-100 (BG00011), which specifically blocks integrin αvβ6, has shown potential as an antifibrotic medication [51]. A phase II study with STX-100 (NCT00878761) administered to individuals with chronic allograft dysfunction, however, resulted in discontinuation for unknown reasons.

To avoid adverse events caused by a complete blockade of TGF-β, selective blockers of the TGF-β downstream signaling pathway have attracted increasing attention. Further basic research and clinical trials are required to discover a precise approach to TGF-β inhibition.

**Potential application of extracellular vesicles in renal fibrosis therapy**

Increasing evidence suggests that pathological signaling delivered by EVs could be essential for tubulointerstitial communication in renal inflammation and fibrosis [38]. Therefore, inhibition of endogenous damage-associated EVs is
a potential therapeutic strategy. Moreover, the therapeutic functions of MSC-derived EVs have given rise to increasing interest due to their intrinsic contents. Notably, EVs could also act as a natural “Trojan horse” to deliver a variety of drugs to treat kidney diseases. Schematic EV-based therapeutic strategies for renal fibrosis are shown in Fig. 2.

**Inhibition of pathogenic extracellular vesicles**

EVs are secreted by parent cells and travel to neighboring or remote sites to exert their function, a process that could be inhibited by targeting the release and uptake of EVs. Pharmacologically, a number of agents have been demonstrated to inhibit EV secretion through different

![Figure 2. EV-based therapeutic strategies for renal fibrosis.](image)

(A) Due to the pathological effects of EVs in renal inflammation and fibrosis, inhibition of EV secretion or uptake is a potential strategy for kidney diseases. (B) EVs derived from stem cells or healthy renal intrinsic cells could act as direct natural therapeutics. EVs can also be used as delivery vehicles for a variety of drugs, including nucleic acids, proteins, and small molecules. EV-based treatments have shown therapeutic effects on renal fibrosis through inhibition of apoptosis, inflammation, and fibrosis and promotion of autophagy, angiogenesis, and proliferation.

EVs, extracellular vesicles; EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; IL, interleukin; miRNA, microRNA; mRNA, messenger RNA; MSCs, mesenchymal stem cells; MVB, multivesicular body; RBC, red blood cell; srkB, super-repressor IκB; TECs, tubular epithelial cells.
mechanisms. Manumycin A, the most widely used farnesyltransferase inhibitor, is necessary for exosome synthesis in the endosomal sorting complex required for transport (ESCRT)-dependent pathway and has been shown in an in vitro model to block exosome release to support the repair of damaged renal epithelium [52,53]. GW4869 is a blockade of neutral sphingomyelinase (nSMase), which mediates ESCRT-independent intraluminal vesicle formation, and has been widely reported as a pharmacological agent to inhibit exosome release in various cancers as well as kidney diseases. Reduced exosome secretion after GW4869 treatment inhibited fibroblast activation and ECM deposition in unilateral IRI in mice [54]. Phosphatidylinerine externalization plays a crucial role in membrane budding and formation of microvesicles (MVs). The pantothenic acid derivative pantethine was demonstrated to impair MV release by preventing the transfer of phosphatidylinerine. In an experimental model, mice treated orally with D-pantethine showed alleviation of fibrosis, and such a protective function might be associated with reduction of endothelial-derived MVs in circulation [55]. Calpain could be activated by calcium to regulate cytoskeleton remodeling and then increase MV release. As a calpain inhibitor, calpeptin treatment could reduce bleomycin-induced pulmonary fibrosis by inhibiting EMT-related markers and the TGF-β1 signaling pathway, which might be related to MV reduction [56].

EVs are significant intercellular communication mediators that interact with recipient cells in an autocrine, paracrine, or endocrine manner [57] through three mechanisms: membrane fusion, receptor (direct) interaction, and internalization [58]. Hence, they may provide an alternative method to inhibit exosome function by blocking uptake. Heparan sulfate proteoglycans (HSPGs) participate in the internalization of cancer cell-derived exosomes, which depend on intact HSPG synthesis and HS sulfation in target cells. Heparin as an HS mimetic inhibits exosome uptake dose dependently [59]. In addition, Bonsergent et al. [60] found that EV uptake is a slow process by quantification analysis, and content delivery can be inhibited by bafilomycin A1 and IFIMT protein overexpression in a pH-dependent manner.

Due to the heterogeneity of EVs and recipient cells, further investigation is warranted to clarify the precise mechanism of formation and uptake of EVs for developing precise therapeutic strategies targeting pathogenic EVs.

**Utilization of mesenchymal stem cell-extracellular vesicles as therapeutic agents**

Remarkable therapeutic effects of anti-inflammation, anti-fibrosis, and prorogeneration have been demonstrated by MSCs for treatment of kidney disease. However, there are safety issues related to immune responses, toxicity, and carcinogenicity [61]. MSCs function in a paracrine or endocrine manner and are coordinated by seoretomes, growth factors, cytokines, and EVs [62]. Compared to MSCs, EVs derived from MSCs are characterized by higher safety, lower immunogenicity, easier preservation, and genetic stability.

A growing number of studies are exploring the potential properties of MSC-EVs in CKD models. Intravenous administration of EVs derived from bone marrow MSCs (BM-MSC) ameliorated tubular necrosis and interstitial fibrosis and improved renal function in a mouse model of aristolochic acid-induced nephropathy [63]. EVs originated from BM-MSCs and human liver stem-like cells also attenuated fibrosis in diabetic nephropathy due to modulation of fibrosis-related gene expression by miRNA cargoes [64]. In a clinical pilot study involving 40 CKD patients with eGFR between 15 and 60 mL/min/1.73 m², both intravenous and intraarterial administration of cell-free MSC-EVs from umbilical cord blood regulated immune response and improved renal function [65]. EVs containing miR-26a-5p from adipose-derived MSCs protected against diabetic nephropathy by targeting toll-like receptor 4 [66]. Additionally, exosomes of human umbilical cord MSCs have been shown to promote nuclear YAP shuttle to cytoplasm and to reduce matrix accumulation by transporting casein kinase 1 δ and β-TRCP to target cells [67]. Interestingly, human urine-derived stem cell exosomes protected against diabetic nephropathy by reducing apoptosis of podocytes and enhancing angiogenesis and cell survival [68].

In conclusion, MSC-EVs may become an alternative therapeutic tool for kidney disease, and further research should be conducted to promote the transition of MSC-EVs to clinical application.

**Extracellular vesicles as therapeutic delivery vehicles**

As natural membrane structures, EV capacity to transfer biomolecules to recipient cells has attracted considerable attention for its potential to overcome limitations of lipo-
**Table 1. Therapeutic applications of EVs in kidney disease**

<table>
<thead>
<tr>
<th>Functional agent</th>
<th>EV origin</th>
<th>Animal model</th>
<th>Efficacy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleic acid</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>miR-34c-5p</td>
<td>BM-MSC</td>
<td>UUO</td>
<td>Reduced pericyte, fibroblast, and macrophage activation and renal fibrosis</td>
<td>[74]</td>
</tr>
<tr>
<td>miR-186-5p agomir</td>
<td>MSC</td>
<td>UUO</td>
<td>Inhibited ECM accumulation and EMT process</td>
<td>[76]</td>
</tr>
<tr>
<td>miR-125a</td>
<td>AD-MSC</td>
<td>DN</td>
<td>Reduced mesangial hyperplasia, expansion of mesangial matrix, and kidney fibrosis</td>
<td>[77]</td>
</tr>
<tr>
<td>miR-26a-5p</td>
<td>HEK293 cell</td>
<td>UUO</td>
<td>Suppressed muscle wasting and renal fibrosis by targeting FoxO1 and CTGF</td>
<td>[78]</td>
</tr>
<tr>
<td>let-7i-5p antagomir</td>
<td>BM-MSC</td>
<td>UUO</td>
<td>Reduced renal fibrosis by activating the TSC1/mTOR pathway</td>
<td>[79]</td>
</tr>
<tr>
<td>miR-16-5p</td>
<td>Urine-derived stem cells</td>
<td>DN</td>
<td>Improved diabetic nephropathy and inhibited podocyte apoptosis by reducing VEGF-A</td>
<td>[80]</td>
</tr>
<tr>
<td>miR-29</td>
<td>Primary mouse satellite cell</td>
<td>UUO</td>
<td>Ameliorated skeletal muscle atrophy and attenuated kidney fibrosis</td>
<td>[81]</td>
</tr>
<tr>
<td>miR-20b-3p</td>
<td>AD-MSC</td>
<td>Ethylene glycol-induced hyperoxaluria</td>
<td>Reduced cell autophagy and inflammatory responses</td>
<td>[82]</td>
</tr>
<tr>
<td>miR-let7c</td>
<td>BM-MSC</td>
<td>UUO</td>
<td>Attenuated kidney injury and reduced ECM accumulation and fibrotic-related gene expression</td>
<td>[83]</td>
</tr>
<tr>
<td>siP65 and siSnai1</td>
<td>Red blood cell</td>
<td>I/R-induced AKI or UUO</td>
<td>Alleviated tubulointerstitial inflammation and fibrosis and abrogated the transition to CKD</td>
<td>[75]</td>
</tr>
<tr>
<td>Oct-4 mRNA</td>
<td>UC-MSC</td>
<td>I/R</td>
<td>Increased the therapeutic effects of MSC-EVs to attenuate kidney fibrosis</td>
<td>[84]</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
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<tr>
<td>Klotho</td>
<td>Urine/fibroblast</td>
<td>AKI generated by glycerol injection</td>
<td>Accelerated renal recovery, stimulated tubular cell proliferation, and reduced inflammation; reduced renal retention and tissue injury; promoted amelioration of renal function</td>
<td>[85]</td>
</tr>
<tr>
<td>IL-10 protein</td>
<td>RAW264.7 cell</td>
<td>I/R-induced AKI</td>
<td>Ameliorated renal tubular injury and inflammation and prevented AKI-to-CKD transition</td>
<td>[73]</td>
</tr>
<tr>
<td>GDNF</td>
<td>AD-MSC</td>
<td>UUO</td>
<td>Ameliorated peritubular capillary loss in tubulointerstitial fibrosis</td>
<td>[86]</td>
</tr>
<tr>
<td>Super-repressor IkBα</td>
<td>HEK293T cell</td>
<td>CLP-induced sepsis</td>
<td>Attenuated mortality, acute organ injury, and inflammation by inhibiting the NF-κB pathway</td>
<td>[87]</td>
</tr>
<tr>
<td>Super-repressor IkBα</td>
<td>HEK293T cell</td>
<td>I/R-induced AKI</td>
<td>Alleviated renal damage and ameliorated inflammation and apoptosis</td>
<td>[88]</td>
</tr>
<tr>
<td>CD26</td>
<td>TCMK1 cell</td>
<td>I/R-induced AKI</td>
<td>Protected against kidney injury by maintaining proliferation and dissipating inflammation</td>
<td>[89]</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Kidney MSC</td>
<td>Model of CKD and renal anemia</td>
<td>Improved hemoglobin levels and renal function in CKD mice and exerted antifibrotic and anti-inflammatory effects</td>
<td>[90]</td>
</tr>
<tr>
<td><strong>Small molecule</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>RAW264.7 cell</td>
<td>LPS- or ADR-induced nephropathy</td>
<td>Suppressed renal inflammation and fibrosis without apparent glucocorticoid adverse effects</td>
<td>[72]</td>
</tr>
</tbody>
</table>

AD, adipose mesenchymal stem cell; ADR, adriamycin; AKI, acute kidney injury; BM, bone marrow; CKD, chronic kidney disease; CLP, cecal ligation and puncture; CTGF, connective tissue growth factor; DN, diabetic nephropathy; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; EV, extracellular vesicle; GDNF, glial-derived neurotrophic factor; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; mRNA, messenger RNA; MSC, mesenchymal stem cell; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; UC, umbilical cord; UUO, unilateral ureteral obstruction; VEGF-A, vascular endothelial growth factor A.
somes and other synthetic drug delivery systems. EV-based therapies offer many outstanding advantages due to the natural lipid structure and modifiable membrane properties achieved through manipulation of parent cells to improve stability as well as by targeting specific tissues and cells [69].

Endogenous and exogenous loading are two principal approaches to integrate therapeutic drugs into exosomes [70]. The technical methodology can be found in another published review [71]. Here, we summarize the studies regarding application of EVs as a therapeutic agent delivery system in renal disease (Table 1) [72–90].

Molecular drugs
EVs have been extensively used as delivery vectors for small molecules in recent studies. Curcumin carried by exosomes had excellent biological function in terms of solubility, stability, and bioavailability. Curcumin encapsulated by exosomes exerted anti-inflammatory effects in lipopolysaccharide-induced brain inflammation and myelin oligodendrocyte glycoprotein peptide-induced experimental autoimmune encephalomyelitis [91]. Additionally, exosome-loaded doxorubicin showed less accumulation in off-target tissues and was less cardiotoxic than unmodified doxorubicin [92,93].

Dexamethasone-loaded macrophage-derived MVs exhibited superior anti-inflammatory and antifibrotic activity without apparent glucocorticoid adverse effects [72], suggesting the possibility of EVs for drug transfer in renal disease.

Therapeutic proteins
Exosomes have an intrinsic capacity to cross biological barriers. Macrophage-derived exosomes without modification can penetrate the blood-brain barrier to transfer brain-derived neurotrophic factor to the central nervous system [94]. Recently, we successfully constructed IL-10-loaded EVs by engineering macrophages to target the injured kidney, which significantly improved renal tubular injury and inflammation and prevented the transition to CKD [73]. Concerning the stability and validity of large molecular cargoes, however, there are technical obstacles to efficiently load proteins into EVs. Increasing attempts had been made to solve this problem; for example, Leidal et al. [95] successfully loaded RNA-binding proteins into EVs via LC3-conjugation machinery.

Genetic materials
Natural EVs can carry both coding RNAs (mRNAs) and noncoding RNAs (long noncoding RNAs, miRNAs, and circular RNAs), which suggests outstanding ability in transferring diverse RNAs for therapeutic purposes [96]. Particularly, miRNAs in engineered EVs have been widely studied in kidney diseases. For example, BM-MSC-derived exosomes inhibited core fucosylation by delivering miR-34c-5p to reduce activation of pericytes, fibroblasts, macrophages, and renal interstitial fibrosis [74]. Furthermore, miRNAs loaded in EVs could be applied for personalized tumor vaccines [97] and the coronavirus disease 2019 pandemic [98]. In addition, EVs loaded with small interfering RNA (siRNA) could also be a promising strategy to mediate gene silencing for cancer therapy [99]. Engineered red blood cell-derived EVs modified with peptide targeting KIM-1 have been constructed and successfully delivered siRNAs against P65 and Snai1 into injured kidneys. Dual inhibition of P65 and Snai1 expression significantly alleviated kidney inflammation and fibrosis in mouse models of IRI and UUO [75].

Conclusion and perspectives
In recent years, it has been shown that maladaptive repair of TECs, PTC rarefaction, activation and proliferation of myofibroblasts, diverse functions of macrophages, ECM hemostasis, and EV-mediated cellular communications are important in tubulointerstitial inflammation and fibrosis. However, the accurate mechanism of renal fibrosis remains to be fully clarified. By combination and integration of single-cell and multiomics techniques, it is now possible to better understand disease mechanisms [100–102]. In addition, EV-mediated cellular communication also provides a new insight into the pathogenesis process of renal fibrosis.

Despite the development of therapeutic agents ranging from chemical compounds to gene therapies against renal fibrosis, clinical translation from bench to bedside is often limited due to the slow progression of disease and heterogeneity of patients as well as lack of noninvasive biomarkers for renal fibrosis [103]. Recent research showed that positron emission tomography imaging of collagen and molecular imaging of fibrosis may allow efficient, noninvasive, quantitative, and longitudinal results [104]. Urinary
EVs released from intrinsic renal cells hold the potential to predict and monitor CKD progression as a “fluid biopsy” approach; for instance, miR-29 in urine EVs has been identified as a biomarker of renal fibrosis [105].

EV-based treatments are promising approaches to realize targeted therapy. However, clinical application of EVs in kidney disease is far from practical. Low production of EVs greatly hinders their clinical application, which encourages advances in stimulating EV shedding and production, but the properties and functions of EVs should be evaluated further. In addition, engineered EVs, including EVs from genetically engineered cells, post-modified EVs (drug loaded, surface modified), and EV-inspired liposomes have been developed to enhance therapeutic activity [106]. MSC-EVs and EVs from other sources have been tested for safety and efficacy in numerous ongoing clinical trials for diseases including diabetes, SARS-CoV-2 pneumonia, Alzheimer’s disease, and various tumors [106]. Due to the heterogeneity of EVs, it is urgent to establish a good unified standard to achieve large-scale and efficient production of clinical-grade EVs for clinical application. Therapeutic EV production is a complex process in which minor changes can have significant impacts on product quality and efficacy [106]. Furthermore, administration route, dosage, and biological distribution of EVs in vivo should be considered before EV products are applied in patients. The underlying mechanisms of EV production, secretion, and uptake are not fully clarified at present. Robust studies of basic EV biology are needed to enable clinical translation. Emerging advanced technologies such as super resolution microscopy, single extracellular vesicle assay, and nanoflow cytometry could be useful tools to achieve deep understanding of EV biology [107].

Overall, with progress in understanding the mechanisms of renal fibrosis as well as the emerging therapeutic strategies, particularly EV-based therapies, we look forward to a new era of precise and targeted treatments for renal fibrosis.

**Conflicts of interest**

All authors have no conflicts of interest to declare.

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**Data sharing statement**

The data presented in this study are available on request from the corresponding author.

**Authors’ contributions**

Conceptualization: CW, SWL, LLL
Funding acquisition, Supervision: BCL, LLL
Writing–original draft: CW, SWL
Writing–review & editing: All authors
All authors read and approved the final manuscript.

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Diminazene aceturate exacerbates renal fibrosis after unilateral ureteral obstruction in female mice

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Background: Diminazene aceturate (DIZE), an angiotensin-converting enzyme 2 (ACE2) activator, exerts anti-inflammatory and antifibrotic effects in a variety of human chronic diseases. However, the role of DIZE in kidney fibrosis and the underlying mechanism remain unclear. Therefore, we investigated the effects of DIZE on the progression of renal fibrosis after unilateral ureteral obstruction (UUO), a well-established model of chronic kidney disease.

Methods: C57BL/6 female or male mice were subjected to right UUO. Mice received 15 mg/kg DIZE or vehicle (saline) daily. On the 7th day after UUO, kidneys were collected for analysis of renal fibrosis (α-smooth muscle actin, phosphorylated SMAD3, transforming growth factor (TGF)-β, Masson’s trichrome, and Sirius red staining), inflammation (macrophage infiltration, proinflammatory cytokines/chemokines), apoptosis/necrotic cell death (TUNEL and periodic acid-Schiff staining), and ACE2 activity and messenger RNA (mRNA) expression.

Results: Treatment with DIZE exacerbated renal fibrosis by upregulating the profibrotic TGF-β/SMAD3 pathway, proinflammatory cytokine/chemokines (interleukin [IL]-1β, monocyte chemoattractant protein-1, IL-6, and macrophage inflammatory protein-2) levels, M2 macrophage accumulation (CD206, IL-4, IL-10, and CX3CL1), and apoptotic/necrotic cell death in the obstructed kidneys of female mice but not male mice. However, DIZE treatment had no effect on ACE2 activity or mRNA expression.

Conclusion: DIZE exacerbates UUO-induced renal fibrosis by aggravating tubular damage, apoptosis, and inflammation through independent of angiotensin (1–7), angiotensin II levels, and ACE2 expression/activity, rather than protecting against renal fibrosis after UUO. DIZE also has powerful effects on recruiting macrophages, including the M2-polarized subtype, in female UUO mice.

Keywords: Chronic kidney diseases, Diminazene aceturate, Inflammation, Kidney fibrosis, M2 macrophage

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**Introduction**

Chronic kidney disease (CKD) is a common, global, progressive disease and a severe clinical problem associated with high mortality and morbidity [1]. Furthermore, there are no ideal drugs for the treatment or prevention of renal fibrosis, which is a major cause of the development and progression of CKD. In addition, renal fibrosis, regardless of its cause, is the final common stage in almost all CKDs [2]. Renal fibrosis is characterized by increased numbers of myofibroblasts, infiltration and accumulation of inflammatory cells, and excessive accumulation of extracellular matrix (ECM) components such as collagen and fibronectin [3].

In rodents, the unilateral ureteral obstruction (UUO) model is widely used to study progressive renal fibrosis because pathological features of CKD, such as tubular dilation, interstitial expansion, loss of tubular mass, leukocyte infiltration, tubular epithelial cell death, and the accumulation of myofibroblasts, are present in this model [4]. Pathogenically, hemodynamic changes are caused by mechanical stretching, apoptosis of epithelial tubular cells, oxidative stress, and inflammation, all of which contribute to progressive renal tubulointerstitial fibrosis [5]. The origins of renal myofibroblasts have been investigated over the last decade as the key reactive cells in renal fibrogenesis. Identifying the origins of renal myofibroblasts may elucidate the diverse characteristics and behaviors of myofibroblasts, which in turn could aid in the development of therapeutics for the treatment and prevention of CKD [6].

Current evidence strongly suggests that collagen-producing myofibroblasts in the kidney originate from a variety of cellular sources, including bone marrow-derived cells, pericytes, resident fibroblasts, macrophages, and epithelial cells [7].

Diminazene aceturate (DIZE; C₁₄H₁₅N₇·2C₂H₅NO₃) is an aromatic diamidine that was developed more than six decades ago and which is currently marketed for the control of trypanosomiasis [8]. DIZE is also a well-known activator of angiotensin-converting enzyme 2 (ACE2), which converts angiotensin II to angiotensin (1–7) [9,10], and several reports have demonstrated that angiotensin (1–7) attenuates the development of cardiac fibrosis [11], renal fibrosis [12], pulmonary fibrosis [13], and hepatic fibrosis [14]. Besides being an ACE2 activator, the drug’s beneficial effects may be ACE2-independent in many pathological conditions [15]. Indeed, it has been suggested that DIZE exerts anti-inflammatory and antifibrotic activities in many human chronic disease models through angiotensin (1–7) independently of ACE2 expression/activity or angiotensin levels. For example, Rajapaksha et al. [16] demonstrated that DIZE protects against liver injury and biliary fibrosis by ameliorating proinflammatory cytokine levels, cellular oxidative pathways, and profibrotic cytokine expression without affecting ACE2 expression/activity or angiotensin levels. Similarly, cardiac fibrosis and diastolic dysfunction were ameliorated by DIZE treatment without affecting ACE2 activity [17]. However, the role of DIZE in kidney fibrosis and the underlying mechanism remain unclear. Therefore, we investigated the effects of DIZE on the progression of renal fibrosis after UUO in mice.

**Methods**

**Surgical preparation of animals**

All experiments were conducted using 8-week-old C57BL/6 mice (male and female) weighing 20 to 25 g each. The study was approved by the Institutional Animal Care and Use Committee of Pukyung National University (No. PKNUIACUC-2021-49) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 2011). Mice were allowed free access to water and standard mouse feed. Animals were anesthetized with pentobarbital sodium (60 mg/kg body weight, intraperitoneal injection [i.p.]; Sigma-Aldrich) before surgery. To induce ureteral obstruction, the right kidney was exposed via a flank incision, the right ureter was completely obstructed using a 6-0 silk thread, and the incision was subsequently sutured [18]. Some mice were intraperitoneally injected with DIZE (15 mg/kg body weight, i.p.; Sigma-Aldrich) daily for 7 days after surgery. The dosage of DIZE was based on previous studies [10,19]. Body temperature was maintained at 36.5–37 °C throughout all surgical procedures using a temperature-controlled heating device (FHC, Inc.). Seven days after surgery, the kidneys were excised and snap-frozen in liquid nitrogen for molecular analysis or perfusion-fixed in 4% paraformaldehyde (Sigma-Aldrich) for immunostaining and histological studies.
**Western blotting**

Western blot analyses were performed as described previously [18] using the following antibodies: anti-\(\alpha\)-smooth muscle actin (\(\alpha\)-SMA, 1:10,000 dilution; Sigma-Aldrich), anti-phosphorylated SMAD3 (p-SMAD3, 1:2,000 dilution; Abcam), nuclear factor kappa B (NF-\(\kappa\)B, 1:2,000 dilution; Cell Signaling), and Ly6G (1:2,000 dilution; eBioscience). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10,000 dilution; Bioworld Technology) was used as a protein loading control. ImageJ software 1.53e was used to quantify band densities.

**Real-time polymerase chain reaction**

Messenger RNA (mRNA) was extracted from kidney samples using TRIzol solution (Ambion). mRNA was reverse transcribed into complementary DNA using M-MLV reverse transcriptase (Promega). Faststart Universal SYBR Green Master Mix (Roche) was used as the quantitative real-time (qRT)-polymerase chain reaction (PCR) reaction mix. We used CFX Connect Real-Time PCR Detection System (Bio-Rad). Sequences of sense and antisense primers used in the murine qRT-PCR are as follows:

- For TGF-\(\beta\): 5’-TTGTACGCGCAGTGGCTGAAC-3’ and 5’-AGTTGGTATCAGGGCTCTCC-3’
- For MCP1: 5’-CCAAGGGTTGATCAGGGCTCTCC-3’ and 5’-TTGTACGCGCAGTGGCTGAAC-3’
- For TGF-\(\beta\): 5’-ACCTGCTGCTACTCATTCAC-3’ and 5’-TTGAGGTGGTTGTGGAAAAG-3’
- For MIP2: 5’-CCAAGGGTTGATCAGGGCTCTCC-3’ and 5’-TTGTACGCGCAGTGGCTGAAC-3’
- For IL-1\(\beta\): 5’-ACCTGGTAGAAGTGATGCC-3’ and 5’-GAGAAATCGATGACAGCGCC-3’
- For IL-4: 5’-CTGGCTTGCTCATCCGCAT-3’ and 5’-GGAGAGCATTGGAAATTG-3’
- For CX3CL1: 5’-TGATGGCATTAGGGCTCTCCAT-3’ and 5’-TGGCCGATCTGCTTTTACCTC-3’
- For PAI-1: 5’-TTGTACGCGCAGTGGCTGAAC-3’ and 5’-AGTTGGTATCAGGGCTCTCC-3’
- For IL-6: 5’-TTGTACGCGCAGTGGCTGAAC-3’ and 5’-AGTTGGTATCAGGGCTCTCC-3’
- For ACE2: 5’-ACCACTGCATGCGCATTAC-3’ and 5’-CACCACTGGTTGCTGAGGCC-3’ for GAPDH.

**Sirius red staining and Masson’s trichrome staining**

Paraffin-embedded kidney tissue sections were stained to detect collagen deposition using the Picro-Sirius Red Stain Kit (Abcam) and Masson’s trichrome stain [20]. For picrosirius red staining, deparaffinized sections were covered completely with picrosirius red solution for 1 hour. Samples were then rinsed twice with acetic acid solution (Sigma-Aldrich). For Masson’s trichrome staining, deparaffinized sections were refixed in Bouin’s solution for 1 hour at 56 °C, and sections were stained in Weigert’s iron hematoxylin working solution for 10 minutes. After washing, sections were stained in Biebrich scarlet-acid fuchsin solution for 10 to 15 minutes, followed by immersion in phosphomolybdic-phosphotungstic acid solution for 15 minutes. Picrosirius red or Masson’s trichrome-stained sections were then dehydrated in a graded dilution series of ethanol. Finally, sections were mounted on coverslips with Permount (Fisher Scientific). Micrographs were taken randomly of cortical areas in 200× microscope image fields using a Leica DM2500 microscope (Leica Microsystems GmbH). Areas of collagen accumulation in stained kidney tissues were analyzed using ImageJ software.

**Immunohistochemical staining**

Immunohistochemical (IHC) staining was performed to confirm the expression of \(\alpha\)-SMA, a marker of fibrosis, infiltration of macrophages using an antibody against F4/80, a marker of macrophages, and CD206, an M2 macrophage marker. Oxidative stress was assessed by staining for 8-OHdG, a biomarker of DNA oxidative damage. Paraffin-embedded kidney tissue sections were dehydrated, followed by antigen retrieval, peroxide quenching, and blocking. Then, kidney sections were incubated with primary antibodies in a humid chamber overnight at 4 °C. Primary antibodies against F4/80 (1:100; Bio-Rad), \(\alpha\)-SMA (1:400; Sigma-Aldrich), NF-\(\kappa\)B (1:200; Cell Signaling), CD206 (1:200; Abcam), and 8-OHdG (1:500; Abcam) were used. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rat immunoglobulin G (IgG), HRP-conjugated goat anti-mouse IgG, or HRP-conjugated goat anti-rabbit IgG (Bethyl-Laboratories). Hematoxylin was used to stain nuclei. Sections were observed using a Leica DM2500 microscope. Micrographs of microscope
image fields in cortical areas were taken randomly at 200× and 400× magnification. α-SMA-, F4/80-, CD206-, and NF-κB-positive cells were counted and recorded using a counting tool. The density of 8-OHdG was measured by Fiji ImageJ software as described in a previous study [21].

**Terminal deoxynucleotidyl transferase dUTP nick-end labeling assays**

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were performed using the Dead-end Fluorometric TUNEL System Kit (Promega) according to the manufacturer’s instructions. Briefly, 4-μm kidney sections were deparaffinized and rehydrated. Sections were then incubated with TUNEL reagent mixture for 30 minutes at room temperature and then washed three times with phosphate-buffered saline (5 minutes for each wash). Sections were mounted on coverslips with an antifade mounting medium. Images of microscope image fields in cortical areas were obtained randomly at 200× using a Leica DM2500 microscope. TUNEL-positive cells were counted and recorded in five fields per kidney.

**Histology (periodic acid-Schiff staining)**

Kidney paraffin sections were stained with periodic acid-Schiff (PAS; Muto Pure Chemicals) stain according to a standard protocol. To determine morphological damage to tubular cells, five randomly chosen microscopic fields of the cortex region of each kidney were analyzed using the following scoring method: 0, no damage; 1, mild damage with rounding of epithelial cells and dilated tubular lumen; 2, moderate damage with flattened epithelial cells, dilated lumen, and congestion of lumen; and 3, severe damage with flat epithelial cells lacking nuclear staining and lumen congestion, as described previously [18].

**Enzyme-linked immunosorbent assays to measure angiotensin (1–7) and angiotensin II**

Blood was collected from the inferior vena cava using heparin sodium salt (20 IU/mL; Bioworld). Plasma was obtained by centrifuging the blood samples at 8,000×g for 5 minutes at 4 °C. Kidney tissue was dissolved in the radio-immunoprecipitation assay lysis buffer using homogenizer and kidney sample was obtained by centrifuging the kidney lysates at 16,000×g for 20 minutes at 4 °C and transferring supernatant to fresh tubes. Enzyme-linked immunosorbent assays (ELISAs) of plasma and kidney samples were performed using an angiotensin (1–7) ELISA Kit (Novus Biologicals) and angiotensin II ELISA kit (Cusabio) according to the manufacturers’ instructions.

**Statistical analysis**

Data were analyzed using Student t tests, one-way analysis of variance, and Tukey *post hoc* multiple comparison tests. Results are expressed as mean ± standard error of the mean. Statistical significance was set at p < 0.05.

**Results**

**Diminazene aceturate exacerbates unilateral ureteral obstruction-induced renal interstitial fibrosis in female mice**

To explore whether DIZE attenuates fibrosis in the UUO kidney, we first investigated α-SMA protein expression, a myofibroblast marker [22], by western blotting. UUO significantly increased α-SMA protein expression compared to that in control kidneys. DIZE treatment after UUO did not affect α-SMA protein expression or the number of α-SMA–positive cells compared to vehicle treatment in male mice (Fig. 1A, B, E, F). However, in female mice, DIZE further increased α-SMA protein expression and α-SMA–positive cells in UUO kidneys compared to vehicle treatment (Fig. 1G, H, K, L). To further investigate the underlying mechanism, we examined whether DIZE affected the transforming growth factor (TGF)-β1/SMAD3 signaling pathway because TGF-β1 is a key mediator of fibrosis development and progression in CKD patients through SMAD3 phosphorylation [23]. SMAD3 phosphorylation and TGF-β1 mRNA expression were increased in female UUO kidneys compared to control kidneys, but DIZE treatment had no effect on SMAD3 phosphorylation or TGF-β1 mRNA expression in male mice (Fig. 1A, C, D). By contrast, SMAD3 phosphorylation and TGF-β1 mRNA expression were higher in DIZE-treated UUO female mice than in vehicle-treated UUO female mice (Fig. 1G, I, J). There were no significant differences in α-SMA expression, SMAD3 phosphorylation, or TGF-β mRNA expression between
**Figure 1.** DIZE exacerbates UUO-induced fibrosis in the kidneys of female mice. Mice were subjected to right unilateral ureteral obstruction. Male (A–F) or female (G–L) mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, (A, G) kidney samples were subjected to western blotting using α-SMA and p-SMAD3 antibodies, which are markers of fibrosis. GAPDH was used as a loading control. (B, C, H, I) Bands densities were quantified using ImageJ software. (D, J) Levels of TGF-β mRNA, a master regulator of renal fibrosis, increased after UUO; DIZE treatment further significantly increased TGF-β mRNA levels in female mice but not in male mice. Kidney sections were subjected to immunohistochemical staining using an antibody against α-SMA. Representative images of α-SMA IHC staining (E, K) are shown and the number of α-SMA–positive cells (F, L) were counted. Results are expressed as mean ± standard error of the mean (n = 6).

α-SMA, α-smooth muscle actin; Con, control; DIZE, diminazene aceturate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemical; mRNA, messenger RNA; phosphorylated SMAD3, p-SMAD3; TGF, transforming growth factor; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney, †p < 0.05 vs. vehicle-treated ureteral obstructed kidney.
vehicle-treated and DIZE-treated control kidneys (Fig. 1). Contrary to our original hypothesis, these results indicate that DIZE exacerbates UUO-induced renal interstitial fibrosis in female mice but not in male mice, suggesting that sex may play a critical role in determining the effects of DIZE on renal fibrosis after UUO.

Next, we investigated whether DIZE affected collagen deposition in UUO kidneys of female mice using Sirius red staining and Masson’s trichrome staining [20]. UUO increased collagen deposition, and DIZE treatment further increased this deposition compared to the vehicle treatment (Fig. 2).

**Diminazene aceturate aggravates unilateral ureteral obstruction-induced histological tubular damage and apoptotic cell death in kidneys**

Next, we investigated if DIZE affects tubular cell damage and cell death. As expected, UUO-induced tubular damage is characterized by formation of proteinaceous casts and increased tubular dilation and congestion. DIZE treatment aggravated this damage in female mice (Fig. 3A). Moreover, we confirmed that UUO increased apoptotic cell death, as evaluated by TUNEL staining, and that DIZE treatment further increased apoptotic cell death in female mice (Fig. 3B). Taken together, these results indicate that DIZE exacerbates UUO-induced histological tubular damage and apoptotic cell death in the kidneys of female mice.

**Diminazene aceturate treatment has no effect on angiotensin-converting enzyme 2 activity or angiotensin (1–7) and angiotensin II levels after unilateral ureteral obstruction**

Because DIZE is a well-known activator of ACE2, which converts angiotensin II to angiotensin (1–7) [9,10], we assessed ACE2 activity by measuring angiotensin II and angiotensin (1–7) levels as well as ACE2 mRNA expression in kidneys (Fig. 4). Consistent with previous studies [24], we found that angiotensin II and angiotensin (1–7) protein levels, as well as ACE2 mRNA expression in kidneys, were significantly increased after UUO. However, DIZE treatment did not affect the UUO-induced changes in ACE2 mRNA expression or angiotensin II and angiotensin (1–7) protein levels in kidneys. These results suggest that DIZE exacerbates UUO-induced renal fibrosis independently of ACE2 after UUO in female mice.

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**Figure 2. DIZE exacerbates UUO-induced collagen deposition in the kidneys of female mice.** Mice were subjected to right unilateral ureteral obstruction. Female mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, kidney sections were subjected to Sirius red staining (A) and Masson’s trichrome staining (B). Collagen deposition and Masson’s trichrome positive area were measured as described in the Methods section. (A, B) Representative images of kidney sections subjected to Sirius red and Masson’s trichrome staining are presented. Results are expressed as mean ± standard error of the mean (n = 6).

Con, control; DIZE, diminazene aceturate; UUO, unilateral ureteral obstruction.

* $p < 0.05$ vs. vehicle-treated control kidney, $^\dagger p < 0.05$ vs. vehicle-treated ureteral obstructed kidney.
Figure 3. DIZE aggravates UUO-induced histological tubular damage and apoptotic cell death in female mouse kidneys. Mice were subjected to right unilateral ureteral obstruction. Female mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, kidney sections were subjected to PAS staining (A) and TUNEL staining (B). Representative images of kidney sections subjected to PAS staining and TUNEL staining are presented. Kidney damage was scored as described in the Methods section. Results are expressed as mean ± standard error of the mean (n = 6).

ACE2, angiotensin-converting enzyme 2; Con, control; DIZE, diminazene aceturate; ND, not detected; PAS, periodic acid-Schiff; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney, †p < 0.05 vs. vehicle-treated ureteral obstructed kidney.

Figure 4. DIZE treatment does not affect ACE2 mRNA expression or protein levels of angiotensin II and angiotensin (1–7) in UUO female mice. Female mice were subjected to right unilateral ureteral obstruction. Mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, mRNA expression of ACE2 in the kidney was measured (A). (B–D) Protein levels of angiotensin II and angiotensin (1–7) in plasma or kidneys were measured using ELISA kits. Results are expressed as mean ± standard error of the mean (n = 6).

ACE2, angiotensin-converting enzyme 2; Con, control; DIZE, diminazene aceturate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney.

Diminazene aceturate aggravates inflammation and infiltration of macrophages in ureteral-obstructed kidneys

Given our finding that angiotensin (1–7) level had no relationship with DIZE-induced aggravation of renal fibrosis after UUO, we hypothesized that DIZE aggravates proinflammatory signaling and inflammatory cell accumulation in UUO kidneys, resulting in increased renal fibrosis. To
test this hypothesis, we assessed the accumulation of inflammatory cells and levels of proinflammatory cytokines/chemokines. Proportion of F4/80-positive cells increased after UUO, and the increase in these cells was greater in DIZE-treated mice than in vehicle-treated female mice after UUO (Fig. 5A, B). We also confirmed that the protein expression of Ly6G, a neutrophil marker, was slightly but significantly increased after UUO. However, treatment with DIZE had no effect on Ly6G protein expression in UUO kidneys or control kidneys compared with that in vehicle-treated female mice (Fig. 5C). In addition, we also determined that macrophage infiltration and levels of cytokines/chemokines, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), interleukin (IL) 1β, and IL-6 were increased after UUO, and that the increases in MCP-1, MIP-2, IL-1β, and IL-6 were greater in UUO kidneys of female mice treated with DIZE than in those of control mice (Fig. 6A–D). We also confirmed activation of the major proinflammatory NF-κB pathway using western blotting and IHC staining (Fig. 6E–G, I). UUO substantially increased the expression of NF-κB, and NF-κB expression was further increased by DIZE treatment. In addition, 8-OHdG, a biomarker of DNA oxidative damage, was increased after UUO, and DIZE treatment further increased levels of this biomarker (Fig. 6H, J).

**Diminazene aceturate aggravates profibrotic M2 macrophage accumulation after unilateral ureteral obstruction**

Acute tissue injury is caused by accumulation of proinflammatory M1 macrophages and the fibrotic response is driven by the persistent presence of profibrotic M2 macrophages [25]. M2 macrophages promote kidney fibrosis by producing excessive ECM and secreting profibrotic growth factors. DIZE exacerbates UUO-induced renal fibrosis, and DIZE treatment further increases the proportion of M2 macrophages in UUO kidneys compared with control kidneys.

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**Figure 5. DIZE aggravates infiltration of macrophages in the ureteral obstructed kidneys of female mice.** Female mice were subjected to right unilateral ureteral obstruction. Mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, kidney samples were subjected to immunohistochemical staining using an antibody against F4/80, a macrophage marker. (A, B) Representative images of F4/80 IHC staining are shown. Seven days after surgery, kidney samples were subjected to western blotting using antibodies against Ly6G, which is a marker of neutrophils (C). GAPDH was used as a loading control. Band densities were quantified using ImageJ software. Results are expressed as mean ± standard error of the mean (n = 6).

Con, control; DIZE, diminazene aceturate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemical; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney; †p < 0.05 vs. vehicle-treated ureteral obstructed kidney.

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Figure 6. DIZE aggravates inflammation in ureteral obstructed kidneys of female mice. Female mice were subjected to right unilateral ureteral obstruction. Mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, kidney samples were subjected to a quantitative real-time polymerase chain reaction. The expressions of proinflammatory cytokines and chemokine mRNAs in the kidney were measured (MCP-1 [A]; MIP-2 [B]; IL-1β [C]; interleukin-6, IL-6 [D]). Seven days after surgery, kidney samples were subjected to western blotting using antibodies against NF-κB, a marker of inflammation (E, F). GAPDH was used as a loading control. Band densities were quantified using ImageJ software. Kidney sections were subjected to immunohistochemical staining using antibodies against NF-κB (I) or 8-OHdG (J). Number of NF-κB-positive cells (G) and density of 8-OHdG staining (H) were measured. Results are expressed as mean ± standard error of the mean (n = 6).

Con, control; DIZE, diminazene aceturate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemical; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; mRNA, messenger RNA; NF-κB, nuclear factor kappa B; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney, †p < 0.05 vs. vehicle-treated ureteral obstructed kidney.
factors. As we had determined that DIZE aggravated the infiltration of total macrophages after UUO (Fig. 5), and because it was recently suggested that DIZE shifts macrophage polarization toward the profibrotic M2 phenotype [26], we evaluated M2 macrophage accumulation by IHC staining. In addition, we measured levels of cytokines and chemokines involved in M2 macrophage survival and polarization. Using qRT-PCR, we found that mRNA expression of CX3CL1, IL-4, and IL-10, which are involved in M2 macrophage survival and polarization, increased after UUO, and DIZE treatment further increased the mRNA expression of these cytokines after UUO (Fig. 7A–C). In addition, the proportion of cells positive for CD206, an M2 macrophage marker [27], increased after UUO, and DIZE treatment further increased M2 macrophage accumulation after UUO (Fig. 7D).

**Discussion**

We originally hypothesized that DIZE treatment would protect against renal fibrosis after UUO. Our original hypothesis was based on the following previous findings: 1) DIZE is a well-known ACE2 activator that converts angiotensin II to angiotensin (1–7), which has antifibrotic and anti-inflammatory properties in diverse tissues, including the heart [11], kidney [12], lung [13], and liver [14]; 2) DIZE exerts anti-inflammatory and antifibrotic activities in many human chronic disease models via angiotensin (1–7) independently.

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**Figure 7. DIZE induces M2 macrophage polarization.** Female mice were subjected to right unilateral ureteral obstruction. Mice were treated with DIZE or vehicle as described in the Methods section. Seven days after surgery, kidney samples were subjected to quantitative real-time polymerase chain reaction to detect mRNA expression of CX3CL1 (A), IL-4 (B), and IL-10 (C). Kidney sections were subjected to immunohistochemical staining using an antibody against CD206, an M2 macrophage marker. (D) Representative images of CD206 IHC staining are shown and the number of CD206-positive cells was counted. Results are expressed as mean ± standard error of the mean (n = 6).

Con, control; DIZE, diminazene aceturate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemical; IL, interleukin; mRNA, messenger RNA; UUO, unilateral ureteral obstruction.

*p < 0.05 vs. vehicle-treated control kidney, †p < 0.05 vs. vehicle-treated ureteral obstructed kidney.
of ACE2 expression/activity or angiotensin levels; 3) the renin-angiotensin system (RAS), which is regulated by DIZE, is a critical regulator of renal fibrosis. To our surprise, we found that mice treated with DIZE had exacerbated renal fibrosis, inflammation, and M2 macrophage accumulation after UUO. Therefore, we suggest that DIZE has a detrimental role in renal fibrosis induced by UUO, rather than a protective role.

RAS regulates fluid balance, blood pressure, and maintains vascular tone. RAS is active in multiple tissues and organs, including the endothelium, myocardium, kidney, and brain. RAS consists of two pathways: the classical and non-classical pathways [9]. In the nonclassical axis, ACE2 converts angiotensin II into angiotensin (1–7), a heptapeptide that stimulates the Mas oncogene receptor (MasR) expressed in blood vessels. Numerous experimental studies have shown that angiotensin (1–7) exerts a vasoprotective role by increasing nitric oxide bioavailability, possibly through MasR, resulting in improved vasodilation [10]. In a diabetic mouse model, DIZE activated ACE2 and increased angiotensin (1–7) levels. These increased angiotensin (1–7) levels exerted a protective effect on diabetic mice by blocking the RAS system [10]. However, in the UUO mouse model, we and others [24,28] have found that angiotensin II and angiotensin (1–7) protein levels as well as ACE2 mRNA expression are significantly increased after UUO. DIZE treatment did not affect the UUO-induced changes in ACE2 mRNA expression or angiotensin II and angiotensin (1–7) protein levels in the kidney (Fig. 4). These results suggest that DIZE exacerbates UUO-induced renal fibrosis independently of ACE2 after UUO in female mice. Numerous studies have shown that kidney ACE2 activity as well as ACE2 mRNA and protein expressions are sex-dependent with higher ACE2 activity and expression in males than females [29–31]. In this study, we found that DIZE treatment after UUO did not affect renal fibrosis as assessed by α-SMA protein expression, SMAD3 phosphorylation, and TGF-β mRNA induction in male mice (Fig. 1A–F). Based on these findings, we speculate that the detrimental effect of DIZE on M2 macrophage-mediated renal fibrosis progression and the protective effects of DIZE on ACE2 activation may compensate for each other. However, how DIZE protects against renal fibrosis progression in UUO male mice remains to be defined in future studies.

Myofibroblasts are reactive cells with the combined characteristics of fibroblasts and smooth muscle cells. They are found in the presence of damage or under pathological conditions, such as cancer and human fibrotic diseases. α-SMA, which produces bundles of myofilaments termed stress fibers, is a myofibroblast-specific marker. Stress fibers connect the myofibroblast to the ECM; when the cell contracts, mechanical stresses are exerted on the matrix, triggering reorganization and wound closure throughout the healing process [18]. The majority of research has attributed this phenomenon to TGF-β, which performs its biological function by activating the SMAD3 signaling (p-SMAD3) pathway downstream and plays a vital role in the development of fibrosis. TGF-β activation controls a variety of cellular activities such as proliferation, apoptosis, differentiation, and inflammation. Furthermore, it stimulates fibroblast proliferation and ECM formation, resulting in profibrotic activity. In this study, we found that DIZE treatment markedly exacerbated UUO-induced renal interstitial fibrosis, activation of TGF-β/SMAD3 signaling, and myofibroblast accumulation (Fig. 1), indicating that DIZE plays a detrimental role in renal fibrosis by upregulating the profibrotic pathway.

Monocytes/macrophages are derived from bone marrow progenitors and enter the bloodstream. Circulating monocytes leave the bloodstream and enter tissues during homeostasis and inflammation. Following exposure to local growth factors, proinflammatory cytokines and microbial products induce the development of monocytes into macrophages [32]. In the early stages of CKD, macrophages infiltrate the renal cortex and increase the production of proinflammatory cytokines (IL-6, IL-1β, and TNF-α), reactive oxidative species such as superoxide anions and oxygen and nitrogen radicals, and matrix metalloproteinases; the increased production of these factors allows macrophages to migrate through basement membranes and interstitial ECM networks [33]. TNF-α attracts monocytes and macrophages to the tubulointerstitium of UUO kidneys by inducing upregulation of MCP-1 expression. In the present study, we found that macrophage production or levels of attracting cytokines/chemokines such as MCP-1, MIP-2, IL-1β, and IL-6 as well as macrophage infiltration were significantly increased by DIZE treatment compared to vehicle in UUO mice (Fig. 5, 6).

The monocyte-macrophage lineage can be activated by various stimuli and classified as either classically activated
Acute tissue injury is caused by proinflammatory M1 macrophages, and the fibrotic response is driven by the persistent presence of profibrotic M2 macrophages. In CKD, accumulated M2 macrophages promote kidney fibrosis by producing excessive ECM and secreting profibrotic growth factors. For example, M2 macrophages secrete TGF-β, which contributes to the differentiation of progenitor cells into myofibroblasts and serve as a source of cytokines and growth factors with fibrogenic properties. They also secrete proteases, which participate in matrix remodeling. Diverse factors can modulate M2 macrophage differentiation, recruitment, and survival. IL-4 and IL-10 polarize M2 macrophages by activating STAT6 and STAT3 via the IL-4 receptor (IL-4R) and the IL-10 receptor, respectively. CX3CL1, also known as fractalkine, is expressed by renal tubular cells as a membrane-bound molecule, and CX3CL1 produced locally has the potential to promote CKD development by attracting CX3CR1-expressing M2 macrophages and fibrocytes into the kidneys. A recent study demonstrated that DIZE increased α-SMA expression and promoted M2 macrophage polarization in an apolipoprotein E-knockout (apoE−/−) mouse model by stabilizing atherosclerotic lesions. In this study, we also found that DIZE treatment aggravated M2 macrophage accumulation after UUO and increased the mRNA expression of CX3CL1, IL-4, and IL-10. In addition, the number of CD206 (M2 macrophage marker)-positive cells was increased by DIZE treatment after UUO, suggesting that DIZE treatment exacerbates UUO-induced renal interstitial fibrosis, activation of TGF-β/SMAD3 signaling, and myofibroblast accumulation, at least in part, by M2 macrophage induction.

In summary, we demonstrated in this study that DIZE exacerbated UUO-induced renal fibrosis by aggravating tubular damage, apoptosis, and inflammation via angiotensin (1–7) independently of ACE2 expression/activity or angiotensin levels, rather than protecting against renal fibrosis after UUO in female mice. In particular, DIZE had a powerful macrophage-recruiting effect, including recruitment of the M2-polarized subtype, in UUO kidneys. Our findings suggest that therapeutic strategies with effects antagonistic to that of DIZE may help attenuate CKD and that treatment of kidney diseases should not focus solely on the previously reported therapeutic effects of DIZE.

Conflicts of interest
All authors have no conflicts of interest to declare.

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Data sharing statement
The data presented in this study are available on request from the corresponding author.

Authors’ contributions
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Upregulation of NADH/NADPH oxidase 4 by angiotensin II induces podocyte apoptosis

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Background: Angiotensin II induces glomerular and podocyte injury via systemic and local vasoconstrictive or non-hemodynamic effects including oxidative stress. The release of reactive oxygen species (ROS) from podocytes may participate in the development of glomerular injury and proteinuria. We studied the role of oxidative stress in angiotensin II-induced podocyte apoptosis.

Methods: Mouse podocytes were incubated in media containing various concentrations of angiotensin II at different incubation times and were transfected with NADH/NADPH oxidase 4 (Nox4) or angiotensin II type 1 receptor for 24 hours. The changes in intracellular and mitochondrial ROS production and podocyte apoptosis were measured according to the presence of angiotensin II.

Results: Angiotensin II increased the generation of mitochondrial superoxide anions and ROS levels but suppressed superoxide dismutase activity in a dose- and time-dependent manner that was reversed by probucol, an antioxidant. Angiotensin II increased Nox4 protein and expression by a transcriptional mechanism that was also reversed by probucol. In addition, the suppression of Nox4 by small interfering RNA (siRNA) reduced the oxidative stress induced by angiotensin II. Angiotensin II treatment also upregulated AT1R protein. Furthermore, angiotensin II promoted podocyte apoptosis, which was reduced significantly by probucol and Nox4 siRNA and also recovered by angiotensin II type 1 receptor siRNA.

Conclusion: Our findings suggest that angiotensin II increases the generation of mitochondrial superoxide anions and ROS levels via the upregulation of Nox4 and angiotensin II type 1 receptor. This can be prevented by Nox4 inhibition and/or antagonizing angiotensin II type 1 receptor as well as use of antioxidants.

Keywords: Angiotensin II, Angiotensin type 1 receptor, Apoptosis, NADPH oxidases, Oxidative stress, Podocytes
described in vascular smooth muscle cells and endothelial cells, is induction of oxidative stress that may arise from activation of NADH/NADPH oxidase (Nox) [6–8], an enzyme that is also present in renal cells such as mesangial cells [9], podocytes [10,11], and proximal tubular epithelial cells [12]. Nox4 is the most abundant isoform of the Nox proteins in podocytes and promotes podocyte injury in diabetic nephropathy [13]. It has been well documented that excessive reactive oxygen species (ROS) can cause protein oxidation, lipid peroxidation, and DNA damage, leading to cell damage and apoptosis [14]. Ang II mediates apoptosis as well as oxidative stress via Ang II type 1 receptor (AT1R) in the kidney [2,3].

Podocytes play a critical role in maintaining the permeselective function of glomerular filtration units [15,16]. The release of ROS causes proteinuria by affecting glomerular endothelial cells, podocytes, and basement membrane and by disturbing normal glomerular permeability [17,18]. The podocyte is a terminally differentiated cell, and severe injury to the cell causes apoptosis without regeneration [4,5].

In this study, using an in vitro model of cultured podocytes, we questioned whether Ang II induces oxidative stress in podocytes and investigated the underlying molecular mechanisms to determine whether such oxidative stress may induce podocyte apoptosis.

**Methods**

**Mouse podocyte culture**

Conditionally immortalized mouse podocytes were kindly provided by Peter Mundel (Goldfinch Bio) and were cultured and differentiated as described previously [19]. Briefly, to stimulate podocyte proliferation, cells were grown at 33 °C (growth permissive conditions) in RPMI 1640 medium in the presence of 10 U/mL mouse recombinant γ-interferon (Roche). Subsequent experiments were performed with differentiated cells. For podocytes to acquire a differentiated phenotype, they were maintained at 37 °C in 95% air/5% CO2 without γ-interferon (non-permissive conditions) for at least 2 weeks. All experiments were performed at least three times independently.

**Treatment conditions and preparation of antibodies**

Mouse podocytes were treated with Ang II (Sigma Chemical Co.) at the indicated concentrations and time durations in 50 μM of probucol (Sigma Chemical Co.) in 70% ethanol as an antioxidant. Primary antibodies used were goat anti-Nox4 (N-15) antibody (sc-21860; Santa Cruz Biotechnology), goat anti-β-tubulin antibody (sc-9104; Santa Cruz Biotechnology), and polyclonal rabbit anti-rat zonula occludens (ZO)-1 (61-7300; Invitrogen).

**Small interfering RNA for Nox4 and AT1R transfection**

The podocytes were transfected by Nox4, AT1R small interfering RNAs (siRNAs), or negative control scrambled siRNA for 24 hours and incubated in the conditions stated above. One day before transfection, the culture medium was removed, and differentiated podocytes were cultivated in antibiotic-free RPMI 1640 medium supplemented with 10% FBS. Transfection began when cell confluence was 70% to 80%. Transient knockdown of Nox4 or AT1R was performed in mouse podocytes using Lipofectamine 2000 Transfection Reagent (Invitrogen) as per the instructions provided by the manufacturer. Briefly, Nox4 siRNA (sc-41587; Santa Cruz Biotechnology), AT1R siRNA (sc-29751; Santa Cruz Biotechnology), or control scrambled siRNA (Santa Cruz Biotechnology) were diluted into each six-well plate with transfection medium (Opti-MEM; Invitrogen) and incubated for 5 minutes. In parallel, lipofectamine diluted with Opti-MEM and siRNA were mixed, incubated at room temperature for 20 minutes, and then added to the cultured podocytes. After replacing the transfection mixture with RPMI 1640 medium after 5 hours, the inhibitory effect of siRNA was confirmed using western blotting analysis.

**Indices of oxidative stress**

Production of superoxide anion (O2−·) was detected using a modification of the technique based on specific reduction of cytochrome C by superoxide anion in the media [20]. Briefly, after podocytes were exposed to RPMI 1640 without phenol red (GIBCO/BRL) with Ang II or probucol, cytochrome C (0.2 mg/mL, type II; Sigma Chemical Co.) was added to the media, and aliquot volumes of 300 μL were
assayed at 2, 6, 12, or 24 hours of exposure time. The specific reduction of cytochrome C induced by superoxide anion was measured using a spectrophotometer at a wavelength of 550 nm at 25 °C. Results are expressed as % relative to the control (condition without Ang II).

To assay superoxide dismutase (SOD) activity, the podocytes exposed to Ang II or probucol for 6, 12, and 24 hours were washed three times with ice-cold phosphate-buffered saline (PBS) and harvested in SOD assay buffer containing 2-amino-2-methyl-1,3-propanediol, boric acid, and diethylene triamine pentaacetic acid. The cells were disrupted by several freeze-thaw cycles, and the homogenates were clarified via centrifugation at 8,500×g for 10 minutes at 4 °C. Clarified cell culture supernatants can generally be assayed without extraction. Protein content was measured in an aliquot of the homogenate using the method described by Lowry et al. [21]. The SOD activity was measured using BLUE TECH SOD-525 (OxisResearch) and was evaluated in various conditions; the values were compared with those of the control.

**Measurement of reactive oxygen species production**

Intracellular ROS generation was assayed using the cell-permeable fluoroprobe 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA; Molecular Probes). To examine the effect of Ang II on ROS generation, mouse podocytes were stimulated with 10−6-M Ang II for 2 or 24 hours. To determine the effect of probucol or siRNA on Ang II-stimulated ROS generation, the mouse podocytes transfected with or without siRNA were co-treated with 10−6-M Ang II or 50-μM probucol. After treatment, cells were loaded with 10-mM CM-H$_2$DCFDA, examined at 30 minutes using immunofluorescence microscopy, and immediately analyzed with an enzyme-linked immunosorbent assay reader at 480-nm excitation and 530-nm emission wavelengths. The fold changes were expressed as % of the control (condition without Ang II).

Mitochondrial ROS production was measured with MitoSOX Molecular Probes (M36008; Invitrogen), a red fluorescent dye that localizes to mitochondria. After treatment, live cells were washed three times with warm PBS and then incubated with 0.5-μM MitoTracker Green (M7514; Invitrogen) and 5-μM MitoSOX for 30 minutes at 37 °C [22]. Cell fluorescence was observed using inverted immunofluorescence microscopy (Fluoview FV10i; Olympus) at wavelengths of 488 nm for excitation and 594 nm for emission. A MitoSOX index was calculated as MitoSOX intensity per MitoTracker Green-positive area using ImageJ image analysis software (U.S. National Institutes of Health).

For detection of 8-oxo-2′-deoxyguanosine (8-oxo-dG), a major form of oxidatively generated DNA damage, treated cells were fixed in 50% ethanol and then incubated with anti-8-oxo-dG antibody (4354-MC-050; R&D Systems). An 8-oxo-dG index was calculated as immunofluorescence intensity of 8-oxo-dG per individual cell using ImageJ image analysis software [22].

**Immunofluorescence staining**

Podocytes were cultured on cover slides and treated as indicated. The cells were washed twice with cold PBS and fixed for 10 minutes in 4% paraformaldehyde solution, followed by a 10-minute permeabilization in 0.2% Triton X-100 at room temperature. Thereafter, 10% goat serum was applied to block nonspecific binding for 1 hour at room temperature. Cells were then incubated with primary antibodies with a manufacturer-recommended dilution overnight at 4 °C in the dark, followed by a 1-hour incubation with 1:200 (v/v) Alexa Fluor 488 (A32723; Invitrogen) for green and Alexa Fluor 594 (A32758; Invitrogen) for red conjugated secondary antibodies at room temperature. The nuclei were double stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Chemical Co.), and the images were viewed using a fluorescence microscope (TCS SP2 AOBS; Leica). Densitometry analyses of the fluorescence were conducted using Image J.

**Western blotting analysis**

The expression of Nox4 protein was assayed with western blotting analysis. The primary antibodies for anti-Nox4 antibody were diluted for western blotting analysis at 1:1,000. Total proteins were extracted from the podocytes using a protein extraction solution (PRO-PREP; Intron) containing leupeptin as a lysosomal inhibitor as per the manufacturer’s protocols. The extracted protein concentrations were determined using a Bio-Rad kit (Bio-Rad Laboratories); 30 μg of boiled extract was loaded per lane on 10% SDS-
PAGE gels and subsequently transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Thereafter, the membranes were air-dried, blocked in 5% fat-free dried milk, and incubated with the indicated primary antibodies overnight at 4 °C. After three washes with Tris-buffered saline with 0.1% Tween, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature, and the resultant bands were visualized using the enhanced chemiluminescence system (Amersham Biotech Ltd.). To confirm equal loading, blots were reprobed with an anti-β-actin antibody. In each experiment, the ratios of absorbance of each molecule vs. that of β-tubulin were analyzed and expressed as % of control (condition without Ang II).

**Real-time polymerase chain reaction analysis**

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) from the treated podocytes. A total of 2 μg of RNA was reverse-transcribed into complementary DNA (cDNA) with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) as per the manufacturer’s protocol. Real-time quantitative polymerase chain reaction (qPCR) was performed to evaluate the Nox4 messenger RNA (mRNA) level at the following conditions: 95 °C for 30 seconds for annealing and 40 cycles of amplification (95 °C for 15 seconds, 60 °C for 30 seconds). The PCR reaction contained 1× SYBR Green PCR Master Mix (Bio-Rad Laboratories), 1.5 μL of cDNA, and 0.2 μM of Nox4 primers (forward: 5’-CCAGAATGAGGATCCGAGAA-3’, reverse: 5’-ACCAACCTGAAACATGCAA-3’; NM_0053069). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal and loading control (forward: 5’-CGAAGTCAACGGATTTGGTC-3, reverse: 5’-AGCCTTCTCCATGGTGTTGA-3’; NM_008084). The relative mRNA level of Nox4 vs. GAPDH was calculated using the 2^ΔΔCt method, and the fold-change was compared. All real-time qPCR reactions were performed in triplicate with three no-template negative controls for each primer pair, and the values were compared with those of controls.

**Statistical analysis**

The data represent at least three independent experiments. In ROS experiments, more than 30 podocytes in a given field in each independent experiment were examined for each condition. Data are representative images or expressed as mean ± standard deviation. Statistical significance was assessed using nonparametric Kruskal-Wallis analysis of variance analysis or Student t test using the IBM SPSS version 9.0 (IBM Corp.). The p-values of <0.05 were considered statistically significant.

**Results**

**Angiotensin II-induced oxidative stress**

Podocytes were incubated for 2, 6, 12, or 24 hours in media containing 10^{-6}-M Ang II or probucol. Administration of Ang II significantly increased the generation of superoxide anions within 6 hours, and there was a further increase of 63.3% at 24 hours (n = 3) (Fig. 1A; *p < 0.05 and **p < 0.01). However, probucol significantly suppressed Ang II-induced superoxide production (n = 3) (Fig. 1A; *p < 0.05). Administration of Ang II significantly suppressed SOD activity after 12 hours, and that was significantly recovered with probucol (n = 3) (Fig. 1B; *p < 0.05 and †p < 0.05).

Furthermore, we assessed the influence of Ang II on intracellular ROS levels using the CM-H2DCFDA. The podocytes were treated with various concentrations of Ang II (10^{-6}, 10^{-7}, and 10^{-8} M) and incubated for 2 or 24 hours.
Ang II increased ROS levels in dose- and time-dependent manners (n = 3) (Fig. 2A; \(* p < 0.05, \# p < 0.05\), and \(\#\# p < 0.01\)). However, probucol significantly suppressed the intracellular ROS levels at 24 hours (n = 3) (Fig. 2B; \(\# p < 0.01\) and \(\#\# p < 0.01\)).

Based on the ROS levels affected by Ang II in this study and our previous results of endoplasmic reticulum stress (ERS) levels by Ang II [23], we applied 10–6 M of Ang II for subsequent analyses.

Next, we assessed the influence of Ang II on mitochondrial superoxide production using MitoSOX Red with MitoTracker Green and the accumulation of 8-oxo-dG in mitochondrial DNA. Untreated podocytes exhibited very low levels of MitoSOX Red fluorescence when cultured with or without antioxidants, whereas MitoTracker Green fluorescence was detected in cytoplasm (Fig. 2C, D). We quantified the MitoSOX index as MitoSOX intensity per MitoTracker Green-positive area using ImageJ. The quantitative data of MitoSOX index demonstrated that mitochondrial superoxide production was significantly higher in the Ang II-treated condition than in untreated conditions with or without probucol at 24 hours. When Ang II increased mitochondrial superoxide production by more than 2-fold, it was significantly recovered with probucol (n = 6) (Fig. 2C, D; \(* p < 0.05, \# p < 0.01\), and \(\#\# p < 0.01\)). We then examined 8-oxo-dG immunoreactivity and found that cytoplasmic 8-oxo-dG immunoreactivity was significantly increased in the Ang II-treated condition by 2-fold compared with that in the untreated conditions with or without probucol, and that was significantly recovered with probucol (n = 6) (Fig. 2E, F; \(* p < 0.05, \# p < 0.01\), and \(\# p < 0.05\)). Taken together, these data suggest that Ang II induces mitochondrial oxidative stress and oxidative DNA damage in podocytes via downregulation of SOD activity, which is recovered with the antioxidant probucol.
Angiotensin II increases Nox4 expression in podocytes

The distributions of Nox4 and ZO-1 were assessed with indirect immunofluorescence staining in Ang II-treated podocytes at 24 hours of incubation. Nox4 and ZO-1 were co-localized, and $10^{-6}$ M of Ang II appeared to concentrate both molecules and increase the intensity of Nox4 (Fig. 3A), as confirmed with western blotting and real-time PCR analysis.

To determine the effect of Ang II on Nox4 in podocytes, we measured Nox4 protein expression with western blotting and real-time PCR analysis. Density values for Nox4 protein of the representative immunoblots from each group showed a marked increase in Nox4 protein level in Ang II-treated podocytes by 46.9 % at 24 hours after incubation ($n = 4$) (Fig. 3B; $p < 0.01$). However, probucol suppressed the Nox4 protein level significantly in both control and Ang II-treated podocytes (Fig. 3B; $p < 0.05$ and $p < 0.01$, respectively). Additionally, real-time PCR analysis was conducted for Nox4 expression (Fig. 3C). As expected, Nox4 expression was enhanced in Ang II-treated podocytes in a time-dependent manner compared with that in untreated cells ($n = 4$) (Fig. 3C; $p < 0.05$). These results suggest that a transcriptional mechanism contributes to the increase in Nox4 protein in Ang II-treated podocytes.

Figure 2. Ang II-induced generation of mitochondrial oxidative stress in cultured podocytes. (A) Ang II increases the intracellular ROS levels in both dose- and time-dependent manners ($n = 3$); (B) these levels also improve with probucol application ($n = 3$). Magnification, ×200. MitoSOX Red and MitoTracker Green fluorescence (Invitrogen) is localized in cytoplasm (C), and $10^{-6}$ M of Ang II increases the MitoSOX index of MitoSOX intensity per MitoTracker Green-positive area, which is shown as scatter/whisker-box plots (D: $n = 6$). Magnification, ×720; bar = 20 μm. The 8-oxo-dG fluorescence is also localized in cytoplasm (E) and increased by $10^{-6}$ M of Ang II, shown as scatter/whisker-box plots (F: $n = 6$). Nuclei colored blue by DAPI. Magnification, ×720; bar = 20 μm. *$p < 0.05$ and **$p < 0.01$ vs. control and *$p < 0.05$, and **$p < 0.01$ vs. each Ang II-treated condition. Control (100%); compared to conditions without Ang II. All data are presented as mean ± standard deviation.

Ang, angiotensin; DAPI, 4’,6-diamidino-2-phenylindole dihydrochloride; ROS, reactive oxygen species. (Continued to the next page)
Nox4 siRNA inhibits Nox4 protein and subsequent angiotensin II-induced oxidative stress

To evaluate the changes in Nox4 protein and Ang II-induced oxidative stress caused by Nox4 siRNA, we transfected podocytes with Nox4 siRNA. We found that 50 nM of Nox4 siRNA inhibited Nox4 protein by 86% for 24 hours (Fig. 4A). As expected, Nox4 siRNA (50 nM) significantly reduced the Nox4 protein level increased by Ang II (n = 3) (Fig. 4B; p < 0.05). Additionally, Nox4 siRNA (50 nM) significantly reduced the oxidative stress increased by Ang II (n = 4) (Fig. 4C). These results suggest that Ang II promotes podocyte oxidative stress by upregulation of Nox4 in a transcriptional mechanism, and selective inhibition of Nox4 could reduce podocyte oxidative stress in Ang II-treated podocytes.

Angiotensin II induces podocyte apoptosis via oxidative stress and Nox4

We previously found that Ang II-induced podocyte apoptosis in a time- and concentration-dependent manner [5]. In this study, FACS analysis showed that treatment with 10⁻⁶ M of Ang II increased apoptosis significantly (n = 3) (Fig.
Figure 3. Ang II-induced Nox4 expression. (A) Nox4 (green) and ZO-1 (red) co-localized in cytoplasm, and $10^{-6}$ M of Ang II concentrates both and increases the intensity of Nox4. Nuclei colored blue by DAPI. Magnification, $\times400$; bar = 10 μm. Ang II significantly increases Nox4 protein level at 24 hours of incubation in western blotting analysis (B: $n=4$) and Nox4 expression in a time-dependent manner on quantitative polymerase chain reaction analysis (C: $n=4$) in Ang II-treated podocytes. (B) Probucol reduces the Nox4 protein level significantly in control and Ang II-treated podocytes. Data are presented as mean ± standard deviation. *$p < 0.05$ and **$p < 0.01$ vs. control and ##$p < 0.01$ vs. Ang II-treated condition. Control (100%); compared to conditions without Ang II.

Ang, angiotensin; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; Nox4, NADH/NADPH oxidase 4; ZO-1, zonula occludens-1.

5A and B; $p < 0.01$, both), but that increase was mitigated by probucol and Nox4 siRNA ($n=3$) (Fig. 5A and B, respectively; $p < 0.05$, both). These data suggest that Ang II induces podocyte apoptosis via oxidative stress and upregulation of Nox4 that were reduced by application of an antioxidant or selective inhibition of Nox4.
Figure 4. Nox4 inhibition by siRNA mitigates Ang II-induced oxidative stress. (A) Nox4 inhibition by siRNA reduces Nox4 protein as expected, and 50 nM of Nox4 siRNA is a sufficient transfection dose. Nox4 siRNA significantly reduces Nox4 protein level (B: n = 3) and the oxidative stress (C: n = 4) increased by Ang II. Data are presented as mean ± standard deviation. *p < 0.05 and **p < 0.01 vs. control and #p < 0.05, and ##p < 0.01 vs. each Ang II-treated condition. Control (100%); compared to conditions without Ang II. Magnification, ×200.

Ang, angiotensin; Nox4, NADH/NADPH oxidase 4; siRNA, small interfering RNA.

AT1R siRNA inhibits angiotensin II-induced oxidative stress and podocyte apoptosis

To evaluate the role of AT1R in Ang II-induced oxidative stress and apoptosis, we transfected podocytes with AT1R siRNA. We found that 400 nM of AT1R siRNA inhibited AT1R protein by 79% for 24 hours (Fig. 6A), and that Ang II treatment upregulated AT1R protein, which was mitigated by AT1R siRNA (n = 3) (Fig. 6B). Ang II treatment increased oxidative stress and apoptosis as in the previous results (n
that were recovered by AT1R siRNA (n = 3) (Fig. 6C and D, respectively; p < 0.01, both) that recovered AT1R by adding it to Nox4 inhibition could have a better outcome.

Discussion

Ang II mediates podocyte injury via hemodynamic and non-hemodynamic actions, including oxidative stress, ERS, apoptosis, and inflammation, contributing to proteinuria and glomerulosclerosis [1–5, 23]. Severe injuries cause apoptosis of terminally differentiated podocytes, which has

Figure 5. Probucol and Nox4 siRNA reduce Ang II-induced podocyte apoptosis. Ang II induces podocyte apoptosis as determined using fluorescence-activated cell sorting assay, and that is improved with probucol (A) and Nox4 siRNA but not by scrambled siRNA (B). Data are expressed as mean ± standard deviation (n = 3). **p < 0.01 vs. control and *p < 0.05 vs. Ang II-treated condition. Control (100%); compared to conditions without Ang II. Ang, angiotensin; Nox4, NADH/NADPH oxidase 4; siRNA, small interfering RNA.
**Figure 6.** AT1R siRNA inhibits Ang II-induced oxidative stress and subsequent podocyte apoptosis. (A) AT1R inhibition by siRNA reduces AT1R protein, and 400 nM of AT1R siRNA is a sufficient transfection dose. (B) AT1R siRNA significantly reduces AT1R protein upregulated by Ang II (n = 3). Ang II-induced oxidative stress and podocyte apoptosis are improved by AT1R siRNA (C and D, respectively; n = 3). Data are presented as mean ± standard deviation. *p < 0.05 and **p < 0.01 vs. control and #p < 0.05, and ##p < 0.01 vs. each Ang II-treated condition. Control (100%); compared to conditions without Ang II. Magnification, ×200.

Ang, angiotensin; AT1R, angiotensin II type 1 receptor; siRNA, small interfering RNA.
been demonstrated in several in vitro and in vivo models [4,5,11,24–26]. Similar to previous reports, we found that Ang II-induced podocyte apoptosis in vitro [5].

As a non-hemodynamic action of Ang II, induction of oxidative stress contributes to early podocyte ERS and apoptosis and later causes progressive renal injury [23,27,28]. Oxidative stress refers to the imbalance between generation and scavenging of ROS, such as superoxide anion and hydrogen peroxide. The release of free radicals from podocytes may contribute to glomerular injury and proteinuria. Among ROS, superoxide anion, metabolized by SODs, is not only a primary free radical generated by reduction of oxygen but also a source of other oxygen-centered radicals, such as hydrogen peroxide and hydroxyl radical, which participate in lipid peroxidation and induce cellular membrane damage [29]. Ang II decreased the cellular levels of total SOD and induced subsequent podocyte death in a concentration-dependent manner [30]. In the present study, we determined whether Ang II promoted oxidative stress in vitro in podocytes and whether such oxidative stress increased podocyte apoptosis.

Oxidative stress in renal cells, including podocytes, may result from activation of Nox [10,11]. Ang II-induced up-regulation of renal Nox and increased ROS production may participate in the development of hypertension and renal injury [11,27,31,32]. In this study, the suppressed activity of SOD increased Nox4 expression and caused a subsequent increase of >60% in superoxide anion and >2.5 times increase in the intracellular ROS levels in Ang II-treated podocytes at 24 hours. A MitoSOX index was increased and 8-oxod-G was highly accumulated under treatment with Ang II, i.e., Ang II-induced the generation of mitochondrial ROS in cultured podocytes. These findings are significant in that we demonstrated the direct effects of Ang II on the generation of mitochondrial ROS in podocytes and found that increased oxidative stress is related to increased podocyte apoptosis. Moreover, administration of probucol, an antioxidant, decreased Nox4 expression and subsequent oxidative stress and podocyte apoptosis, re-enforcing the role of Ang II in generation of ROS and subsequent podocyte apoptosis.

Ang II mediates oxidative stress and apoptosis via AT1R in the kidney [2,3]. In this study, we found that Ang II treatment upregulated AT1R protein, and inhibition of AT1R by siRNA reduced Ang II-induced oxidative stress and podocyte apoptosis. The reduction of podocyte apoptosis by AT1R siRNA in this study supports our previous findings [5], and the prevention of this by an AT1R antagonist, losartan

**Figure 7. Schematic view of oxidative stress and apoptosis induced by Ang II in a podocyte.** Ang II induces podocyte oxidative stress and subsequent apoptosis with AT1R and via upregulation of Nox4. Ang, angiotensin; AT1R, angiotensin II type 1 receptor; Nox4, NADH/NADPH oxidase 4.
in that case, were also reported recently [11]. These results suggest that Ang II induces podocyte oxidative stress and apoptosis through AT1R and that AT1R inhibition by siRNA and/or antagonizing AT1R could reduce podocyte oxidative stress and apoptosis.

Oxidative stress is considered an initiator and a major contributor to both ERS and autophagy/apoptosis imbalance [33]. In an aldosterone/mineralocorticoid receptor-induced podocyte injury model, ROS driven by aldosterone caused ERS and CHOP-dependent apoptosis and autophagy in cultured podocytes [34]. We previously found that Ang II could induce podocyte ERS of the PERK-eIF2α-ATF4 axis via the PI3-kinase pathway, which was ameliorated by losartan, an AT1R antagonist [23]. Therefore, we aim to understand the role of the ERS pathway in Ang II-induced oxidative stress and subsequent apoptosis to augment the therapeutic potency.

In conclusion, the cumulative data of oxidative stress and apoptosis indicate that Ang II induces excessive ROS production derived from damaged podocyte mitochondria and apoptosis through upregulation of AT1R and Nox4 (Fig. 7). Therefore, Ang II-induced podocyte oxidative stress and subsequent apoptosis through AT1R and Nox4 could be prevented by Nox4 inhibition and/or antagonizing AT1R.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors’ contributions

Conceptualization: TSH, DSH
Methodology: All authors

Writing—original draft: TSH, DSH
Writing—review & editing: All authors
All authors read and approved the final manuscript.

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References


Evaluating the Safety and effectiveness in adult Korean patients treated with Tolvaptan for management of autosomal dominant polycystic kidney disease (ESSENTIAL): short-term outcomes during the titration period

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Background: Tolvaptan reduces height-adjusted total kidney volume (htTKV) and renal function decline in autosomal dominant polycystic kidney disease (ADPKD). This study was aimed at investigating the efficacy and safety of tolvaptan in Korean patients with ADPKD during the titration period.

Methods: This study is a multicenter, single-arm, open-label phase 4 study. We enrolled 108 patients with ADPKD (age, 19–50 years) with an estimated glomerular filtration rate (eGFR) of >30 mL/min/1.73 m² and factors defined as indicative of rapid disease progression. After tolvaptan titration, we evaluated efficacy and side effects and assessed factors associated with the effects.

Results: After titration for 4 weeks, eGFR and htTKV decreased by 6.4 ± 7.9 mL/min/1.73 m² and 16 ± 45 mL/m², respectively. No serious adverse drug reactions were observed during the titration period. The greatest eGFR decline was observed in the first week, with a starting tolvaptan dose of 45 mg. Multivariate linear regression for htTKV decline showed that the greater the change in urine osmolality (Uosm), the greater the decrease in htTKV (β, 0.436; p = 0.009) in the 1D group stratified by the Mayo Clinic image classification. Higher baseline eGFR was related to a higher htTKV reduction rate in the 1E group (β, –0.642; p = 0.009).

Conclusion: We observed short-term effects and safety during the tolvaptan titration period. The decline of htTKV can be predicted as a short-term effect of tolvaptan by observing Uosm changes from baseline to end of titration in 1D and baseline eGFR in 1E groups.

Keywords: Clinical trial phase IV, Polycystic kidney autosomal dominant, Tolvaptan
Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the fourth most common cause of end-stage renal disease worldwide, including Korea [1,2]. The prevalence of ADPKD has been estimated to be 1 per 1,000 individuals [3,4]. ADPKD is also the most common genetic disease of the kidneys, involving the \( \text{PKD1} \) and \( \text{PKD2} \) genes [5]. It affects the kidney and is associated with extrarenal manifestations such as liver cysts and intracranial aneurysms [6,7]. In particular, it is characterized by large fluid-filled kidney cysts caused by increases in arginine vasopressin (AVP) level, resulting in increased intracellular adenosine cyclic monophosphate (cAMP) level in the distal tubule and collecting duct [8]. ADPKD progresses to end-stage renal disease by an average age of 60 years. Sufficient water intake is recommended to lower urine osmolality (Uosm) to 250 mOsm/kg to inhibit increases in cyst size [9]. Tolvaptan is a nonpeptide AVP V2 receptor antagonist known to induce aquaretics by decreasing the concentration of cAMP in the kidney. It has been used for water management in patients with chronic heart failure with hyponatremia and water excretion disorders, such as syndrome of inappropriate antidiuretic hormone [10–12].

Tolvaptan has been used in the management of ADPKD since the positive results of the phase 3 trial, TEMPO (Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and its Outcomes) 3:4, were published in 2013. The TEMPO trial included 1,445 patients having ADPKD (age, 18–50 years) with a total kidney volume (TKV) of 750 mL and estimated creatinine clearance of 60 mL per minute or more. However, the proportion of Asian patients included in the TEMPO 3:4 trial was only 12.6%, and the dosage administered to Japanese participants was lower than the mean dosage for the entire population but was a higher weight-adjusted dosage [13]. Thus, there could be limitations in applying the results of the TEMPO 3:4 trial to Asian patients. Additionally, further considerations are necessary regarding aquaretics-related adverse effects and hepatotoxicity in Asian populations. Therefore, we designed this phase 4 study in a sample of Korean patients with ADPKD to determine the efficacy and safety of tolvaptan. Furthermore, we evaluated short-term effects of tolvaptan on renal function and TKV, quality of life as evaluated using the Pain and Discomfort Scale (PDS) and Urinary Impact Scale (UIS), and the incidence of adverse events during the titration period.

Methods

Ethical considerations

Informed consent was obtained from each patient at the time of enrollment. The study was approved by the institutional review board (IRB) of each participating hospital (representative hospital IRB No. H-1902-041-1009). This study was conducted according to the guidelines of the Declaration of Helsinki. The Clinical Trial registry name and registration number is ESSENTIAL trial (NCT 03949894).

Study population

Patients aged 19 to 50 years who were diagnosed with ADPKD based on the unified criteria for ultrasonography-based diagnosis of the disease were enrolled [14]. Specifically, patients with an estimated glomerular filtration rate (eGFR) of >30 mL/min/1.73 m² and rapid disease progression at the time of screening were included. Rapid progression was defined as a Mayo Clinic image classification (MCIC) of 1C, 1D, or 1E; confirmed presence of a truncating \( \text{PKD1} \) mutation; predicting renal outcome in polycystic kidney disease (PROPKD) score greater than 6 [15]; or rate of decrease in eGFR more than 5 mL/min/1.73 m² within 1 year or 2.5 mL/min/1.73 m² or more per year for 5 years. The exclusion criteria were hyponatremia or hypernatremia, severe hepatic impairment, diabetic nephropathy or any other active glomerulonephritis, a history of hypersensitivity to benzazepine, contraindication of magnetic resonance imaging (MRI), anuria, or poor response to thirst.

Study design

This was a multicenter, single-arm, open-label phase 4 study with screening and tolvaptan titration periods of up to 8 and 4 weeks, respectively. During the titration periods, patients visited hospitals weekly, and the dose was escalated according to the following protocol based on subject tolerability: 45 mg (30 mg + 15 mg) per day for the first week and then 60 mg (45 mg + 15 mg) per day, 90 mg (60 mg + 30 mg) per day.
mg) per day, and 120 mg (90 mg + 30 mg) per day at intervals of at least 1 week during the tolvaptan titration period. During the maintenance period of 48 months, the patients used the highest tolerated dose among 60 mg (45 mg + 15 mg), 90 mg (60 mg + 30 mg), and 120 mg (90 mg + 30 mg) per day. Fig. 1 shows the flow chart of patient inclusion and the entire study design.

Definition of variables and outcomes

The outcomes of this study were changes in renal function, height-adjusted TKV (htTKV), patient-reported outcomes, and adverse events during the first 4 weeks of the study, which was the titration period. The evaluation was performed at the start (0 day) and the end (4 weeks) of the tolvaptan titration period. To evaluate changes in renal function, the serum creatinine level was measured at a central laboratory using the kinetic colorimetry assay, and eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. MRI was performed using a unified protocol. To minimize variability due to the multicenter trial design, each investigating institution designated one MRI scanner and team of technologists to collect trial data. The technologists completed training before the start of the trial, and a central image analysis center qualified all procedures and MRI images per a standardized MRI protocol, including sequences. MR images were acquired by the designated technolo-

![Figure 1. Patient inclusion flow chart and study design.](image)

MRI, magnetic resonance imaging; TKV, total kidney volume; eGFR, estimated glomerular filtration rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
gists following the MRI protocol and de-identified by each investigating institution. After receiving the deidentified MRI images, the image analysis center determined whether the images followed the MRI protocol and measured TKV centrally [16]. htTKV was defined as TKV divided by height. Uosm and creatinine were simultaneously measured at each center. Each patient underwent evaluation using the PDS and UIS during each week of the tolvaptan titration period, and the results were compared at the start (0 day) and the end (4 weeks) of the titration period to assess subjective kidney pain and urinary symptoms [17,18]. The mean prescribed tolvaptan dose was divided by body weight at each visit. Serum Na level was measured and liver function tests for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin were measured to evaluate treatment-emergent adverse events (TEAEs). Adverse events were classified as mild, moderate, or severe. The criteria for classification were as follows: mild, discomfort without disruption to daily life; moderate, discomfort that limits or affects daily activities; and severe, inability to work or perform daily activities.

**Statistical analysis**

Parametric numerical variables were reported as mean ± standard deviation, and median (interquartile range) values were reported for nonparametric numerical variables. Paired t test or Wilcoxon signed-rank test was performed for comparisons before and after tolvaptan administration. The Pearson correlation coefficient was applied to determine the relationships between variables and outcomes. Multivariate linear regression analysis was used to analyze factors influencing htTKV. In the linear regression model, beta (β) was used as a standardized value to correct the units of the variables. Differences were considered statistically significant at p < 0.05.

**Results**

**Baseline characteristics**

Overall, 108 patients (mean age, 38.6 ± 8.5 years) were included in the study, and 57.4% were male. The mean body mass index was 25.2 ± 4.2 kg/m². The mean serum creatinine level and eGFR_{CKD-EPI} were 1.15 ± 0.4 mg/dL and 80.3 ± 27.6 mL/min/1.73 m², respectively. In total, 33.3%, 34.3%, and 31.5% of the subjects were classified as 1C, 1D, and 1E, respectively, according to the MCIC. Stage 3 chronic kidney disease was classified in 42.6% of the patients.

**Table 1. Baseline characteristics of ESSENTIAL trial participants**

<table>
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<tr>
<th>Characteristic</th>
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<tr>
<td>No. of participants</td>
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<tr>
<td>Age (yr)</td>
<td>38.6 ± 8.5</td>
</tr>
<tr>
<td>Male sex</td>
<td>62 (57.4)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.2 ± 4.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>129.4 ± 14.8</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84.0 ± 11.4</td>
</tr>
<tr>
<td>Hypertension</td>
<td>94 (87.0)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>34 (31.5)</td>
</tr>
<tr>
<td>Mayo Clinic image classification</td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>1C</td>
<td>36 (33.3)</td>
</tr>
<tr>
<td>1D</td>
<td>37 (34.3)</td>
</tr>
<tr>
<td>1E</td>
<td>34 (31.5)</td>
</tr>
<tr>
<td>Truncating PKD1 mutation</td>
<td></td>
</tr>
<tr>
<td>PROPKD score, &gt;6</td>
<td>14 (18)*</td>
</tr>
<tr>
<td>Annual eGFR decline, ≥5</td>
<td>3 (3)*</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.25 ± 0.4</td>
</tr>
<tr>
<td>eGFR_{CKD-EPI} (mL/min/1.73 m²)</td>
<td>80.3 ± 27.6</td>
</tr>
<tr>
<td>CKD stage</td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>46 (42.6)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>31 (28.7)</td>
</tr>
<tr>
<td>Stage 3</td>
<td>31 (28.7)</td>
</tr>
<tr>
<td>Serum Na (mmol/L)</td>
<td>140.2 ± 2.5</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>20.3 ± 6.5</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>21.7 ± 14.1</td>
</tr>
<tr>
<td>Urine osmolality (mOsm/kg)</td>
<td>435.9 ± 182.3</td>
</tr>
<tr>
<td>Maximal dose of tolvaptan (mg)</td>
<td>108.0 ± 16.4</td>
</tr>
<tr>
<td>Maximal tolerable dose (mg/day)</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>67 (62.0)</td>
</tr>
<tr>
<td>90</td>
<td>38 (35.2)</td>
</tr>
<tr>
<td>60</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Actual exposed dose (mg)</td>
<td>73.1 ± 5.5</td>
</tr>
<tr>
<td>Weight-adjusted dose (mg/kg)</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Duration of exposure (day)</td>
<td>29.1 ± 2.3</td>
</tr>
</tbody>
</table>

*Data are expressed as number only, mean ± standard deviation, or number (%). PROPKD, predicting renal outcome in polycystic kidney disease; eGFR, estimated glomerular filtration rate; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration; CKD, chronic kidney disease. *The number in parentheses indicates the number of subjects. The titration period was temporarily discontinued in two subjects due to adverse events and maintained at 60 mg and 90 mg, respectively, after resuming the titration period.
nephron disease (CKD) was noted in 28.7% of the patients; the average Uosm at screening was 436 ± 182 mOsm/kg. After the titration period, the mean dose was 73.13 ± 5.5 mg/day; and 62.1%, 35.1%, and 2.8% of the patients took 120 mg/day, 90 mg/day, and 60 mg/day of tolvaptan, respectively (Table 1). The titration period was temporarily discontinued for two subjects due to adverse events, and the respective doses were maintained at 60 mg and 90 mg after the titration period. In subgroups stratified by MCIC, the 1E group was younger (mean age, 32.7 ± 7.6 years; p < 0.001) and had the largest proportion of male participants (88.2%, p < 0.001). In addition, the patients in this group were taller (mean height, 1.75 ± 0.1 m; p < 0.001), heavier (mean body weight, 81.3 ± 17.3 kg; p < 0.001), and received a lower weight-adjusted dose (Supplementary Table 1, available online).

Efficacy and patient-reported outcomes during the titration period

After the 4-week titration period, eGFR significantly decreased by 6.4 ± 7.9 mL/min/1.73 m² (p < 0.001) and htTKV by 16 ± 45 mL/m (p < 0.001) (Table 2, Fig. 2). Percentage changes in htTKV and eGFR did not differ significantly according to MCIC (Supplementary Table 1, available online). Uosm decreased by 264 ± 204 mOsm/kg after 4 weeks of tolvaptan (p < 0.001).

Regarding PDS, a decrease of 0.5 points or more was defined as a meaningful decrease; however, there was no significant decrease after 4 weeks. Regarding UIS, for which the same criteria were applied, frequency (0.81 points), urgency (0.74 points), and nocturia (0.60 points) were significantly increased by tolvaptan (Table 2). During the titration period, the greatest decline of eGFR (−4.6 ± 8.4 mL/min/1.73 m²) was observed during the first week after starting tolvaptan 45 mg (Fig. 3). There was no significant difference in the amount of decrease in eGFR after the first week. This pattern was similar to that associated with CKD 1 progression to CKD 3 when analyzed according to the CKD stage (Supplementary Fig. 1B, available online). Serum Na level showed the greatest increase in the first week, with no subsequent significance. Blood pressure did not change significantly during the entire period.

Associations between variables and outcomes as tolvaptan response

In univariate linear regression analysis, for the entire patient group, the reduction rate of htTKV was predicted by baseline Uosm (β, −0.005; p = 0.003) and change of Uosm

### Table 2. Changes in eGFR, TKV, htTKV, and patient-reported outcomes from baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline (V2)</th>
<th>4 Week (V6)</th>
<th>Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>80.3 ± 27.6</td>
<td>73.9 ± 26.4</td>
<td>−6.4 ± 7.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TKV (mL)</td>
<td>2.070 ± 1.108</td>
<td>2.044 ± 1.119</td>
<td>−27 ± 78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>htTKV (mL/m)</td>
<td>1.213 ± 0.629</td>
<td>1.197 ± 0.635</td>
<td>−16 ± 45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine osmolality (mOsm/kg)</td>
<td>436 ± 182</td>
<td>167 ± 106</td>
<td>−264 ± 204</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Pain and Discomfort Scale**

- **Dull pain severity**: 1.66, 1.45, −0.21, 0.003
- **Sharp pain severity**: 1.19, 1.24, 0.06, 0.27
- **Discomfort severity**: 1.65, 1.74, 0.09, 0.29
- **Overall pain severity**: 1.50, 1.48, −0.02, 0.62
- **Dull pain interference**: 1.31, 1.26, −0.05, 0.37
- **Discomfort interference**: 1.29, 1.40, 0.10, 0.04
- **Sharp pain interference**: 1.12, 1.23, 0.11, 0.03

**Urinary Impact Scale**

- **Frequency**: 1.33, 2.14, 0.81, <0.001
- **Urgency**: 1.27, 2.01, 0.74, <0.001
- **Nocturia**: 1.66, 2.26, 0.60, <0.001

Data are expressed as mean ± standard deviation or points only.

eGFR, estimated glomerular filtration rate; TKV, total kidney volume; htTKV, height-adjusted TKV; V2, visit 2; V6, visit 6.
Figure 2. Percentage change in eGFR and htTKV from baseline. (A) Distribution of percent change from baseline in eGFR$_{\text{CKD-EPI}}$. (B) Distribution of percent change from baseline in htTKV.

eGFR, estimated glomerular filtration rate; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration; htTKV, height-adjusted total kidney volume.

($\beta$, 0.004; $p = 0.02$) (Supplementary Table 2, available online). Additionally, baseline Uosm and change of Uosm after 4 weeks were negatively correlated in the correlation analysis ($r$, $-0.852$; $p < 0.001$) (Supplementary Fig. 1A, available online). In the model adjusted for age, sex, baseline htTKV, eGFR, change of Uosm, and weight-adjusted dose, significant regression was not observed. Considering the heterogeneous characteristics of patients according to
Figure 3. Weekly change in variables during the titration period. (A) Mean change from baseline in eGFR_{CKD-EPI} over time. (B) Mean serum Na over time. (C) Mean systolic blood pressure (SBP) over time. (D) Mean diastolic blood pressure (DBP) over time.
eGFR, estimated glomerular filtration rate; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration.

MCIC (Supplementary Table 1, available online), we performed a subgroup analysis stratified by MCIC. In the subgroup analysis stratified by MCIC, the htTKV reduction rate showed a positive relationship with the decline of Uosm in class 1D ($\beta = 0.436; p = 0.009$) in the multivariable linear regression analysis adjusted for covariates such as age, sex, baseline eGFR, and baseline htTKV (Table 3, Fig. 4A). In class 1E, when all the existing variables were applied, the model did not yield significant results ($F = 1.67; p = 0.20$); therefore, backward elimination was performed (Table 4). Patients with preserved renal function showed a greater decrease in htTKV in class 1E ($\beta = -0.628; p = 0.007$) in the backward elimination model ($F = 2.85; p = 0.03$). The htTKV reduction rate after 4 weeks and baseline eGFR were negatively correlated in class 1E (Fig. 4B). Although there was a prominent decline in eGFR during the first week of the titration period, no correlations were observed in univariate linear regression for factors related to the decline of renal function during the first week (Supplementary Table 3, available online).

Safety and treatment-emergent adverse events during the titration period

We analyzed TEAEs in a total of 117 patients enrolled in the study. No serious adverse drug reactions were observed...
during the titration period. In cases of liver injury, AST and ALT levels were elevated in two patients (1.7%) and indicated mild-to-moderate severity in all patients. After maintaining the tolvaptan dose or temporarily discontinuing tolvaptan, laboratory abnormalities improved. Aquarexis, nocturia, polyuria, and urinary frequency were observed in six (5.1%), 10 (8.5%), and eight cases (6.8%), respectively, and improved in most cases (Supplementary Table 4, available online).

### Discussion

This study aimed to investigate the short-term efficacy and safety of tolvaptan and to identify factors that can predict changes in hTKV during the tolvaptan titration period in patients with ADPKD. We found that a decrease in TKV begins during the tolvaptan titration period, and the greatest decrease in eGFR was observed during the first week of exposure to the initial tolvaptan dose (45 mg/day). Although the decrease in renal function was greatest in the first week of low-dose exposure to tolvaptan, there was no evidence...
Figure 4. Correlation coefficients between variables and percentage change in htTKV. (A) Decline in urine osmolality (Uosm) is proportional to a decrease in htTKV during the titration period in class 1D of Mayo Clinic image classification (MCIC) (n = 37). (B) Baseline eGFR is positively related to the htTKV reduction rate in class 1E of MCIC (n = 34).

htTKV, height-adjusted total kidney volume; eGFR, estimated glomerular filtration rate; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration.

of acidosis or other electrolyte abnormalities requiring discontinuation of tolvaptan through clinical assessment. This finding may be informative for clinicians considering tolvaptan prescription. We also found that the short-term effect of tolvaptan could be predicted through different factors according to MCIC. We observed that a short-term
Uosm change was correlated with decline of htTKV as a response to tolvaptan. Through subgroup analysis, we found that patients classified as 1E with a higher baseline eGFR responded better to tolvaptan. In class 1E, which included younger patients and a larger proportion of male patients, initiating treatment before decline in renal function may be associated with better short-term outcomes in high-risk groups. In addition, most of the adverse events that occurred during the titration period were of mild-to-moderate severity and improved by maintaining or reducing the tolvaptan dose.

Since tolvaptan was initially found to inhibit cyst cell proliferation and cyst growth in ADPKD through *in vitro* and animal experiments, many studies have been conducted in humans [19,20]. Recent studies that demonstrated the long-term effects of tolvaptan are the TEMPO 3:4 and the REPRISE (Replicating Evidence of Preserved Renal Function: an Investigation of Tolvaptan Safety and Efficacy in ADPKD) trials [21,22]. As a phase 3 trial in 1,445 patients for 3 years, TEMPO 3:4 indicated that tolvaptan helped to ameliorate increase in TKV, worsening kidney function, and ADPKD-related composite events [23]. However, the proportion of the trial population made up of Asian patients was small, and there were limitations in the need for titration due to concerns about aquaresis-related adverse events and hepatotoxicity. During the titration period, it is necessary to determine the appropriate tolvaptan dose and whether there are factors that could predict the treatment response in Asians with ADPKD. Decreases in eGFR and kidney cyst size were observed in a 2011 study that reported the effects of tolvaptan administration for 1 week in 20 patients with ADPKD. While renal blood flow was preserved, the tolvaptan-induced decline in renal function was not correlated with baseline renal function [24]. Another study reported that tolvaptan was effective for increasing fractional free-water clearance even in 27 patients with decreased kidney function after 3 weeks of administration [25]. Previous studies have predicted the long-term response to tolvaptan through Uosm [26]. A greater difference in Uosm at baseline and end of titration was associated with better renal outcomes after 3 years in a *post hoc* analysis of the TEMPO 3:4 trial. Uosm was maintained at 200 to 300 mOsm/kg for 3 years and meant by sustained accumulating benefit. Our results also showed that Uosm change correlated with change in htTKV in the short duration of 4 weeks, suggesting its potential as a surrogate marker for predicting short-term treatment response.

Regarding the dose of tolvaptan, a daily split-dose treatment of 90 to 30 mg is currently used in consideration of pharmacokinetic results [27,28]. A previous study showed a positive relationship between weight-adjusted dose and preservation of renal function in Japanese patients [29]. An average dose of 1.14 mg/kg per day was administered, and the follow-up period was 2.52 years. The larger dose was associated with better renal outcomes. In our study, the dose of tolvaptan did not significantly affect short-term outcomes. This finding may be attributable to the difference in the weight-adjusted dose due to the difference in weight and height of the patient group according to the MCIC (1.14 ± 0.22 mg in class 1C; 1.03 ± 0.23 in class 1D; and 0.95 ± 0.21 in class 1E; p = 0.002). With progression in the MCIC, there is a possibility that the exposure dose relative to the actual body weight may decrease in this study population. In the TEMPO 3:4 trial, the protective effect on renal function appeared after 1 year, and in the ALADIN (effect of longacting somatostatin analogue on kidney and cyst growth in autosomal dominant polycystic kidney disease) trial, the greater the rate of decrease in renal function during the first year, the better the renal function after 3 years [30]. This was interpreted to mean that suppression of kidney compensation could be extended for better long-term outcomes. Although the observation periods differed, the pattern of decline in eGFR in the early period of tolvaptan exposure in this study was similar to the pattern of renal function change over several years in previous studies. Further studies of long-term outcomes of the ESSENTIAL trial are necessary to identify whether a decline in eGFR for 4 weeks would have a protective effect on long-term outcomes by inhibiting hyperfiltration in functional nephrons. Furthermore, we found that in 1E patients, the better the baseline renal function, the higher the htTKV reduction rate after 4 weeks. Although the difference was not significant, the preserved baseline renal function was associated with a smaller decrease in eGFR. We suggest that tolvaptan use is necessary before decrease in renal function in cases with preserved kidney function. It is unclear why the factors that predict short-term responses to tolvaptan differ according to MCIC. Depending on the stage of ADPKD, the pathophysiology of disease may be different. ADPKD is accompanied by active proliferation of cysts and inflam-
mation in the parenchymal tissue around cysts in the early stages. As the disease progresses, interstitial fibrosis develops around cysts along with a decrease in eGFR [30]. Given these differences, change in Uosm may be a more valid surrogate marker in 1D, the early stage of the disease. In 1E, the advanced stage, initiating treatment before parenchymal fibrosis progression with eGFR decline may predict a better response.

Our study has some limitations. The first is that the protocol recommends the intake of a sufficient amount of water, but the effect of water intake could not be adjusted using plasma osmolality [31] and total Uosm could not be confirmed in the 24-hour urine test. As the time of urine sampling was variable among patients, it is possible that the pre- or post-dose regimen may have affected Uosm. In general, subjects took tolvaptan at 8 AM and 4 PM, an interval of 8 hours. Allowing modifications of that schedule depending on the subject’s living and sleep patterns may have led to intersubject variation. However, the times and intervals for taking tolvaptan were constant for each subject. Uosm was relatively constant from 4 hours to 16 hours after tolvaptan in the pharmacokinetics and pharmacodynamic study of tolvaptan [28]. The second limitation is that the change of 1.3% in hTKV is close to the range of measurement error in the ellipsoid method. Mean bias of reproducibility between observers was 0.9% in the ellipsoid method [32]. However, a decrease of 1.7% including increased hTKV in this study could be significant over 4 weeks. The third limitation is other potential confounding variables. During the study period, an angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) was administered as a first-line agent in patients with systolic blood pressure of >130 and/or diastolic blood pressure of >80 mmHg to maintain the target blood pressure below 130/80 mmHg. However, medications including ACEis and ARBs could not be adjusted as covariates. Additionally, further analysis of long-term outcomes is needed to address the issue of the tolvaptan dose in ongoing phase 4 trial.

In conclusion, we observed the short-term effects and safety of tolvaptan during a 4-week titration period. We found that the greatest decrease in kidney function occurred during the first week of starting 45 mg of tolvaptan. Short-term effects of tolvaptan could be predicted using different factors according to the Mayo classification. Changes in Uosm may predict the decline of hTKV as a short-term response in 1D, while preservation of renal function before progression to parenchymal fibrosis may be related to better response in 1E, the advanced stage.

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Conflict of interest

Tae-Hyun Yoo is the Editor-in-Chief of Kidney Research and Clinical Practice and was not involved in the review process of this article. All authors have no other conflicts of interest to declare.
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Data sharing statement

The datasets generated and/or analyzed during the current study will be shared on reasonable request to the corresponding author.

Authors’ contributions

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References


The effects of socioeconomic status on major adverse cardiovascular events: a nationwide population-based cohort study

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Background: Although multiple factors influence the risk of major adverse cardiovascular events (MACE), the effects of socioeconomic status on MACE in the presence and absence of renal dysfunction (RD) have not been comprehensively explored in Korea.

Methods: We examined the effects of socioeconomic status on MACE in individuals with and without RD. The data of 44,473 Koreans from 2008 to 2017 were obtained from the Health Care Big Data Platform of the Ministry of Health and Welfare in Korea. Their socioeconomic status was assessed using a socioeconomic score (SES) based on marital status, education, household income, and occupation. The incidence of myocardial infarction (MI), stroke, and death was compared according to SES level (0–4). Multiple linear regression analysis was used to evaluate the hazard ratios and 95% confidence intervals for outcomes based on participant SES.

Results: MI risk was only affected by education level. The participants’ income, education, and SES affected their stroke risk, whereas death was associated with all four socioeconomic factors. The incidence of stroke and death increased as SES worsened (from 0 to 4). SES was positively related to risk of stroke and death in participants without RD. SES did not affect MI, stroke, or death in participants with RD.

Conclusion: A low socioeconomic status is associated with risk of stroke and death, especially in individuals without RD.

Keywords: Death, Myocardial infarction, Renal dysfunction, Stroke
Introduction

Sociologists have long known that social integration [1,2] and socioeconomic status [3] are the strongest predictors of human mortality risk. Equitably delivering high-quality care is an important goal of a high-performing health system. Disparities related to socioeconomic status are of particular concern in the field. Several studies have identified sociodemographic disparities in the incidence of diseases, prevalence of risk factors, and life expectancy [4–7]. In developed countries, a low socioeconomic status tends to increase the risk of cardiovascular disease in men and women [8,9]. However, the results for individual socioeconomic factors have been inconsistent. Moreover, the complex relationships between socioeconomic factors (e.g., household income, education level, marital status, and occupation) and major adverse cardiovascular events (MACE) have not been fully elucidated. Most previous studies have focused on specific socioeconomic factors and not on the combined effects of socioeconomic factors [10].

In addition, simultaneous analysis of the underlying relationships between the complex effects of socioeconomic status and MACE will provide important information on the different pathways through which socioeconomic status, social support, and social network influence public health. Ultimately, the findings of such an analysis would provide evidence that could help in the development and testing of further intervention strategies for reducing public health disparities. Therefore, this study aimed to verify the relationships between mixed socioeconomic factors and MACE in individuals with and without renal dysfunction (RD).

Methods

Study design and database

Public institutions related to health care each store and manage data in their own domains. In Korea, the Health Insurance Review and Assessment (HIRA) service is in charge of drug prescriptions and treatment details, and health checkups are handled by the National Health Insurance Service (NHIS) and the Korea National Health and Nutrition Examination Survey (KNHANES) of the Korea Centers for Disease Control and Prevention. Although large amounts of data are available, it would be very difficult to conduct a comprehensive study linking data dispersed by different institutions. The Ministry of Health and Welfare publicly launched the “Health Care Big Data Platform” in 2020. This platform is owned by the public institutions NHIS, HIRA, Korea Centers for Disease Control and Prevention, and the National Cancer Center to help medical research and policy improvement by linking big data. As the data involve sensitive health and medical information, they are encrypted before transmission on an administrative network between public institutions. To minimize the risk of leakage of personal information, technical (de-identification) measures have been taken to protect and anonymize personal information.

In this study, data from the Health Care Big Data Platform of the Ministry of Health and Welfare in Korea were used. Data from KNHANES 2008–2017 were combined with the medical records and death data from the HIRA and NHIS. The KNHANES consists of health checkups, health interview surveys, and nutrition surveys conducted by trained investigation team members (examiners and interviewers) [11]. A total of 44,473 participants participated in the KNHANES 2008–2017. We excluded 4,772 participants who were <40 years old; 1,768 with previous myocardial infarction (MI); 2,766 with previous stroke; and 4,760 participants with missing data. Thus, data from a total of 30,407 participants were analyzed in this study (Fig. 1).

This study was approved by the Institutional Review Board of Chonnam National University Hospital in Korea (No. CNUH-EXP-2019-299) and by the Institutional Review Board of the National Evidence-based Healthcare Collaborating Agency (No. NECAIRB20-016-1), and informed consent was waived. This study meets the ethical principles of the Helsinki Declaration for medical research involving human participants.

Data collection

Information on household income, education level, marital status, and occupation was collected. Household income was classified into two groups, with the baseline set at the lowest quartile. Education level was classified into two groups: 0–6 years of education (baseline) and >6 years of education. Information on marital status (living with or without a spouse) and occupation status (with or without
Data on general health behaviors, such as current smoking status, alcohol consumption, and physical exercise, were collected using a self-report questionnaire. Smoking status was classified into three categories: never smoker, former (ex-) smoker, and current smoker. Alcohol consumption status was classified into three categories: none, mild, and heavy (drinking ≥30 g/day). Physical exercise was classified into two categories based on a modified form of the International Physical Activity Questionnaire for Koreans [12]: regular walking and non-regular walking. Regular walking was defined as walking more than five times a week for >30 minutes per session.

Participant height, weight, and waist circumference (WC) were measured in casual clothes. Height was measured with an accuracy of 0.1 cm using a portable stadiometer (Seca 225; Seca GmbH), and weight was measured to the nearest 0.1 kg using an electronic scale (GL-6000–20; CASKOREA). WC was measured to the nearest 0.1 cm at the end of expiration at the midpoint of the lower margin of the ribcage and the iliac crest in the participant’s mid-axillary line using a measuring tape (Seca 200; Seca GmbH). Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared (kg/m²) [13].

**Definitions of chronic diseases**

The socioeconomic score (SES) was defined as a score incorporating income, education, occupation, and marital status, ranging from 0 to 4 (Fig. 1). Chronic disease was defined based on a doctor’s diagnosis or a history of treatment for the following diseases: cardiovascular disease (e.g., angina pectoris, MI, and stroke), diabetes mellitus (DM), hypercholesterolemia, and hypertension (HTN). RD was defined as an estimated glomerular filtration rate (eGFR) of <60 mL/min/1.73 m², calculated using KNHANES data in the Modification of Diet in Renal Disease equation [12, 14].

**Study outcomes**

The endpoints of the study were newly diagnosed MI, stroke, or death. MI was defined as International Classification of Diseases, 10th edition (ICD-10) (Supplementary Table 1, available online) codes I21 or I22 during hospitalization or the presence of at least two records of these codes. Stroke was defined by ICD-10 codes I63 or I64 during hospitalization with claims for brain magnetic resonance imaging or brain computed tomography. Although it was difficult to clearly define the stroke subtype (ischemic vs. hemorrhagic), we attempted to exclude cerebral hemorrhage. The study population was followed from baseline to the date of death or cardiovascular event or until December 31, 2018. Participants without MI or stroke during the follow-up period were considered to have completed the study at the date of death or the end of follow-up.
Statistical analysis

Baseline characteristics are presented as mean ± standard deviation or number (percentage). The incidence rate of primary outcomes was calculated by dividing the number of incident cases by the total follow-up duration (person-years). Hazard ratios (HRs) and 95% confidence intervals (95% CIs) for MI, stroke, and death were analyzed using the Cox proportional hazards model for SES. The multivariate-adjusted proportional hazards model was applied, in which model 1 was not adjusted; model 2 was adjusted for age and sex; and model 3 was further adjusted for smoking, alcohol drinking, regular walking, DM, HTN, and dyslipidemia. In subgroup analyses for RD, the HR (95% CI) of SES was compared with SES of 0 as the reference. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc.), and p-value of <0.05 was considered to indicate statistical significance.

Results

Baseline characteristics

Table 1 shows the baseline characteristics of the participants with respect to the occurrence of MI, stroke, and death. In total, 245 participants (0.8%) developed MI, 483 (1.6%) experienced stroke, and 1,517 (5.3%) died. The mean age of the participants who developed MI, experienced stroke, or died was higher than that of those who did not. The proportion of participants with a low income was higher in the event group than in the non-event group. Co-morbidities such as DM, HTN, dyslipidemia, and RD were more prevalent in the event group than in the non-event group. The WC, systolic blood pressure, and glucose levels of the event group were higher than those of the non-event group, but the former’s eGFR was lower. As an outcome, the death group showed a lower BMI than the survival group; however, the event group, including MI and stroke, showed a higher BMI than the non-event group (Table 1).

The characteristics of the participants as per their SES are presented in Table 2. Participants with the highest SES (SES of 4) were older; more likely to be female, nonsmokers, and nondrinkers; exercised less; and displayed a higher prevalence of DM, HTN, dyslipidemia, and RD (Table 2). The blood pressure, fasting glucose, and total cholesterol levels of the highest SES group were higher but their eGFR levels were lower than the reference group (Table 2).

Association of socioeconomic score and risk of myocardial infarction

The lowest income group showed the highest risk of MI. However, it was not statistically significant after adjustment. Education was associated with MI risk, but having an occupation or a spouse did not affect the risk of MI. Finally, SES was not associated with the risk of MI after adjusting for covariates (Table 3). The type of medical insurance also did not affect the development of MI (Supplementary Table 2, available online).

Association of socioeconomic score and risk of stroke

The lowest income group showed the highest risk of stroke after adjusting for covariates. Education was also associated with stroke risk. However, having an occupation or spouse did not affect the risk of stroke. Finally, SES showed a linear relationship with stroke after adjusting for covariates (Table 4). The medical aid group showed a higher HR for stroke compared to groups with other types of medical insurance (Supplementary Table 2, available online).

Association of socioeconomic score and risk of death

All four socioeconomic factors (income, education level, marital status, and occupation) were associated with risk of death after adjusting for covariates. Finally, SES showed a linear relationship with death after adjustment for covariates (Table 5). The risk of death was significantly higher in the medical aid group (Supplementary Table 2, available online).

Effects of renal dysfunction on the associations of socioeconomic score and risk of myocardial infarction, stroke, and death

Subgroup analyses that investigated the effects of RD and smoking history on the association between SES and the risk of MI, stroke, and death were performed. RD did not affect the development of MI after adjusting for covariates (Fig. 2A). SES was associated with risk of stroke and death.
Table 1. Baseline characteristics of subjects according to the incident myocardial infarction, stroke and death

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Myocardial infarction</th>
<th>Stroke</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13,031 (43.2)</td>
<td>12,937 (43.2)</td>
<td>13,296 (42.6)</td>
</tr>
<tr>
<td>Female</td>
<td>17,131 (56.8)</td>
<td>16,987 (56.8)</td>
<td>16,570 (57.4)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>57.3 ± 11.5</td>
<td>57.2 ± 11.4</td>
<td>56.7 ± 11.1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.3 ± 9.3</td>
<td>156.3 ± 9.3</td>
<td>156.4 ± 9.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>57.5 ± 11.0</td>
<td>57.5 ± 11.0</td>
<td>57.7 ± 11.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.0 ± 3.0</td>
<td>24.0 ± 3.1</td>
<td>24.0 ± 3.0</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>77.5 ± 9.5</td>
<td>77.5 ± 9.5</td>
<td>77.5 ± 9.4</td>
</tr>
<tr>
<td>Type of medical insurance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residence</td>
<td>9,400 (31.2)</td>
<td>9,328 (31.2)</td>
<td>9,042 (31.3)</td>
</tr>
<tr>
<td>Worker</td>
<td>19,802 (65.7)</td>
<td>19,662 (65.7)</td>
<td>18,993 (65.7)</td>
</tr>
<tr>
<td>Medical aid</td>
<td>960 (3.2)</td>
<td>934 (3.1)</td>
<td>855 (3.0)</td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>6,102 (20.2)</td>
<td>5,989 (20.0)</td>
<td>5,477 (19.0)</td>
</tr>
<tr>
<td>Q2</td>
<td>7,498 (24.9)</td>
<td>7,438 (24.9)</td>
<td>7,207 (24.9)</td>
</tr>
<tr>
<td>Q3</td>
<td>7,852 (26.0)</td>
<td>7,804 (26.1)</td>
<td>7,656 (26.5)</td>
</tr>
<tr>
<td>Q4</td>
<td>8,710 (28.9)</td>
<td>8,693 (29.1)</td>
<td>8,550 (29.6)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elementary</td>
<td>8,769 (29.1)</td>
<td>8,615 (28.8)</td>
<td>7,973 (27.6)</td>
</tr>
<tr>
<td>Middle</td>
<td>4,232 (14.0)</td>
<td>4,206 (14.1)</td>
<td>4,064 (14.1)</td>
</tr>
<tr>
<td>High</td>
<td>9,576 (31.8)</td>
<td>9,529 (31.8)</td>
<td>9,363 (32.4)</td>
</tr>
<tr>
<td>College</td>
<td>7,585 (25.2)</td>
<td>7,574 (25.3)</td>
<td>7,490 (25.9)</td>
</tr>
<tr>
<td>Spouse, yes</td>
<td>24,790 (82.2)</td>
<td>24,622 (82.3)</td>
<td>23,927 (82.8)</td>
</tr>
<tr>
<td>Occupation, yes</td>
<td>18,943 (62.8)</td>
<td>18,851 (63.0)</td>
<td>18,444 (63.8)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>18,514 (61.4)</td>
<td>18,384 (61.4)</td>
<td>17,910 (62.0)</td>
</tr>
<tr>
<td>Ex</td>
<td>6,114 (20.3)</td>
<td>6,060 (20.3)</td>
<td>5,760 (19.9)</td>
</tr>
<tr>
<td>Current</td>
<td>5,534 (18.4)</td>
<td>5,480 (18.3)</td>
<td>5,220 (18.1)</td>
</tr>
<tr>
<td>Drinker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>9,157 (30.4)</td>
<td>9,050 (30.2)</td>
<td>8,566 (29.7)</td>
</tr>
<tr>
<td>Mild</td>
<td>18,587 (61.6)</td>
<td>18,479 (61.8)</td>
<td>18,040 (62.4)</td>
</tr>
<tr>
<td>Heavy</td>
<td>2,418 (8.0)</td>
<td>2,395 (8.0)</td>
<td>2,284 (7.9)</td>
</tr>
<tr>
<td>Regular walking</td>
<td>11,919 (39.5)</td>
<td>11,816 (39.5)</td>
<td>11,392 (39.4)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>3,658 (12.1)</td>
<td>3,611 (12.1)</td>
<td>3,383 (11.7)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>10,975 (36.4)</td>
<td>10,814 (36.1)</td>
<td>10,299 (35.6)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>5,612 (18.6)</td>
<td>5,581 (18.7)</td>
<td>5,437 (18.8)</td>
</tr>
<tr>
<td>CKD</td>
<td>855 (2.8)</td>
<td>834 (2.8)</td>
<td>701 (2.4)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>121.5 ± 17.2</td>
<td>121.4 ± 17.1</td>
<td>121.2 ± 17.1</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77.2 ± 10.4</td>
<td>77.2 ± 10.4</td>
<td>77.3 ± 10.3</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>101.0 ± 23.7</td>
<td>101.0 ± 23.6</td>
<td>100.8 ± 23.0</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>194.7 ± 36.1</td>
<td>194.7 ± 36.1</td>
<td>195.0 ± 35.9</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>90.3 ± 17.1</td>
<td>90.3 ± 17.1</td>
<td>90.5 ± 16.9</td>
</tr>
</tbody>
</table>

Data are expressed as number (%) or mean ± standard deviation. BMI, body mass index; CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure; TC, total cholesterol; WC, waist circumference.

*Low income 25%, *alcohol consumptions ≥ 30 g/day.
Table 2. Baseline characteristics of subjects according to the social economic status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Social economic status score</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12,219</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3,086</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8,210</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4,069</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2,823</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male</td>
<td>7,433 (60.8)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4,786 (39.2)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>51.2 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>160.4 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61.1 ± 11.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.1 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>77.8 ± 9.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type of medical insurance</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residence</td>
<td>3,819 (31.3)</td>
<td></td>
</tr>
<tr>
<td>Worker</td>
<td>8,346 (68.3)</td>
<td></td>
</tr>
<tr>
<td>Medical aid</td>
<td>54 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Income</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Q1</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>2,814 (23.0)</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>4,125 (33.8)</td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td>5,280 (43.2)</td>
<td></td>
</tr>
<tr>
<td>Education</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Elementary</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>2,073 (17.0)</td>
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</tr>
<tr>
<td>High</td>
<td>5,340 (43.7)</td>
<td></td>
</tr>
<tr>
<td>College</td>
<td>4,806 (39.3)</td>
<td></td>
</tr>
<tr>
<td>Spouse, yes</td>
<td>12,219 (100)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Occupation, yes</td>
<td>12,219 (100)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>6,112 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td>3,083 (25.2)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>3,024 (24.7)</td>
<td></td>
</tr>
<tr>
<td>drinker</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>2,250 (18.4)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>8,575 (70.2)</td>
<td></td>
</tr>
<tr>
<td>Heavy</td>
<td>1,394 (11.4)</td>
<td></td>
</tr>
<tr>
<td>Regular walking</td>
<td>4,638 (38.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1,111 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3,496 (28.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>1,839 (15.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CKD</td>
<td>136 (1.1)</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.6 ± 15.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>78.5 ± 10.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>100.2 ± 22.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>194.4 ± 34.9</td>
<td>0.003</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>91.4 ± 15.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as number only, number (%), or mean ± standard deviation.
BMI, body mass index; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; DBP, diastolic blood pressure; SBP, systolic blood pressure; TC, total cholesterol; WC, waist circumference.

*Low income 25%, *alcohol consumptions ≥ 30 g/day.
### Table 3. IRs and HRs of myocardial infarction according to the socioeconomic status and score

<table>
<thead>
<tr>
<th>Myocardial infarction</th>
<th>No. of subjects</th>
<th>Event</th>
<th>Duration (Person-years)</th>
<th>IR</th>
<th>Model 1 p-value</th>
<th>Model 2 p-value</th>
<th>Model 3 p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Income</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q1</td>
<td>6,191</td>
<td>89</td>
<td>34,674.8</td>
<td>2.57</td>
<td>&lt;0.001</td>
<td>1.35 (0.91–2.01)</td>
<td>1.26 (0.85–1.86)</td>
</tr>
<tr>
<td>Q2</td>
<td>7,564</td>
<td>66</td>
<td>42,014.0</td>
<td>1.57</td>
<td>0.33</td>
<td>1.25 (0.85–1.83)</td>
<td>1.20 (0.81–1.76)</td>
</tr>
<tr>
<td>Q3</td>
<td>7,897</td>
<td>45</td>
<td>43,876.9</td>
<td>1.03</td>
<td>0.52</td>
<td>1.01 (0.67–1.53)</td>
<td>0.99 (0.65–1.49)</td>
</tr>
<tr>
<td>Q4 (highest)</td>
<td>8,755</td>
<td>45</td>
<td>48,186.8</td>
<td>0.93</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
<td>1 (Reference)</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elementary</td>
<td>8,894</td>
<td>125</td>
<td>51,903.2</td>
<td>2.41</td>
<td>&lt;0.001</td>
<td>1.95 (1.23–3.10)</td>
<td>1.84 (1.15–2.94)</td>
</tr>
<tr>
<td>Middle</td>
<td>4,275</td>
<td>43</td>
<td>24,623.7</td>
<td>1.75</td>
<td>0.01</td>
<td>1.82 (1.11–2.97)</td>
<td>1.76 (1.08–2.87)</td>
</tr>
<tr>
<td>High</td>
<td>9,626</td>
<td>50</td>
<td>53,544.9</td>
<td>0.93</td>
<td>0.03</td>
<td>1.27 (0.80–2.04)</td>
<td>1.23 (0.77–1.97)</td>
</tr>
<tr>
<td>College</td>
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<td>27</td>
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CI, confidential interval; HR, hazard ratio; IR, incident rate (per 1,000 person-years); SES, socioeconomic score.

Table 4. IRs and HRs of stroke according to the socioeconomic status and score

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<th>p-value</th>
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CI, confidential interval; HR, hazard ratio; IR, incident rate (per 1,000 person-years); SES, socioeconomic score.

Table 5. IRs and HRs of death according to the socioeconomic status and score

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CI, confidential interval; HR, hazard ratio; IR, incident rate (per 1,000 person-years); SES, socioeconomic score.

Figure 2. Forest plots of subgroup analyses according to the presence or absence of RD or smoking, with the SES 0 group as the reference. (A, D) Risk of myocardial infarction, (B, E) stroke, and (C, F) death. CI, confidence interval; HR, hazard ratio; RD, renal dysfunction; SES, socioeconomic status score.
in participants without RD. However, SES did not affect stroke and death risk in participants with RD (Fig. 2B, C). Smoking was a risk factor for MI, stroke, and death (Fig. 2D–F) but did not affect the association between MI risk and SES (Supplementary Fig. 1D, available online). Stroke risk was associated with SES for nonsmokers but not for smokers (Supplementary Fig. 1E, available online). With regard to death risk, SES was a strong risk factor regardless of smoking status (Supplementary Fig. 1F, available online).

**Discussion**

In the present study, the participants’ level of education was associated with the risk of MI, stroke, and death. The participants’ income level was associated with risk of stroke and death. All four socioeconomic factors were associated with risk of death. SES, a combined measure of the four investigated socioeconomic factors, also showed a relationship with stroke and death. However, the relationships between the effects of SES and risk of stroke or death were attenuated in participants with RD.

Among other sociodemographic factors, lower educational levels have been reported to be associated with limited access to health care, worsening socioeconomic status, and unhealthy lifestyle behaviors [15,16], all of which may considerably contribute to the risk of poor outcomes. Our study also showed that, among the investigated socioeconomic factors, only education level was associated with MI, stroke, and death. Previous data showed that individuals with a primary school education or lower had a 1.7-fold higher incidence of MI in comparison to those with a senior high school, college, or postgraduate education [17]. Studies from Europe and America have reported an inconsistent association between education and adverse cardiovascular outcomes among patients with acute MI based on educational status [7,18–21]. Some of these discrepancies arise from the use of inconsistent assessment methods and inclusion of important cardiovascular risk factors, such as HTN, dyslipidemia, smoking, and preexisting heart diseases, in the assessment of differences [19,20,22]. The mechanisms that support the association between a lower education level and a higher risk of MACE remain unclear. Individuals with higher educational attainment may be more proficient in self-management after discharge and may be more proficient in finding the optimal standard of care [23]. Additionally, they may have better health knowledge and lower financial barriers to access to health care, which may improve their access to follow-up health services. Dedicated studies should focus on the roles of follow-up care, medication adherence, and utilization of rehabilitation services to understand education-based differences in cardiovascular outcomes.

Subsequently, future interventions for less educated individuals could focus on improving the most challenging aspects of post-discharge care and performing a more rigorous follow-up for such vulnerable patients. These findings also stimulate policy and public health discussions, which would facilitate the development of practical and sustainable strategies such as providing targeted populations with more convenient health access and initiating close and active treatment coordination to create appropriate educational materials for them.

A low household income is associated with a variety of indicators of low health status, which include low birth weight, early childhood mortality, and adult mortality [22,24]. Moreover, because individuals with lower income have limited resources, they also have a limited range of food choices or lack the economic ability to engage in health-enhancing activities. Meanwhile, they have been reported to have a high degree of psychosocial stress [25], which increases sympathetic nerve activity and induces left ventricular hypertrophy, resulting in poor chronic kidney disease (CKD) outcomes [26]. As access to health-related activities may be determined by income level, individuals with a higher income are reported to have a higher ability to control their health conditions [27,28].

SES showed a positive relationship with the risk of stroke and death in participants without RD. In contrast, SES did not affect the occurrence of MI, stroke, or death in participants with RD. There are several possible explanations for this phenomenon. As RD is a known powerful risk factor for MACE, and RD patients have a higher prevalence of MACE risk factors such as DM and HTN, individuals with RD may be more influenced by their RD status than by their health habits or other external factors. The prevalence of CKD increases with age, and the difficulty of accurately measuring the income level of older individuals owing to their changing work status and income might have influenced the results in the CKD group [29]. In addition, rel-
atively older participants with CKD may find it difficult to maintain healthy living habits [30]. Subgroup analysis on smoking showed similar results to RD subgroup analysis. In MI and stroke, SES had a significant effect on outcomes in the nonsmokers group, but SES did not significantly affect MI or stroke in the smoking group. These results suggest that the effect of smoking on the occurrence of MI or stroke is higher than that of SES. Therefore, it can be concluded that smoking cessation is necessary to prevent MI and stroke.

Unexpectedly, we did not observe any association between the status of MI and household income or occupation, which contradicts the results of previous studies [31]. Although we do not have a clear explanation for this finding at this time, the wide coverage of the national health insurance and the nationwide management program for coronary artery disease provided by public health centers in Korea might have lowered the barriers to treatment and provided individuals from all walks of life with equal access to treatment for MI. However, further studies that examine various factors that affect MI outcomes (e.g., treatment modality, adherence to treatment, and medical cost) are warranted.

The results of this study should be interpreted with cognizance of its various limitations. First, this study utilized household income, education, occupation, and marital status as indices to represent socioeconomic status. However, these indices may be insufficient for precise evaluation of socioeconomic status. Second, the small event size in the RD group might have introduced an unreliability bias. Third, participants living in rural areas had lower access to healthcare services than those living in cities, but we did not account for the features of the local communities to which the participants belonged. Last, the measurement of serum creatinine using the isotope dilution mass spectrometry-traceable method was introduced in Korea during the research data extraction period from 2008 to 2017. As a result, the serum creatinine measurement method and the eGFR calculation formula may differ across institutions.

Although the prediction of outcomes with a single socioeconomic indicator may be insufficient, there has been no attempt to predict outcomes as a composite indicator by tying these indicators together. As the results show, death and MI were better predicted by the composite variables. A weakness of this study is that, although each indicator may have different effects, they were all combined to acquire a single score.

In conclusion, low socioeconomic status is associated with increased risk of stroke and death, especially in individuals without RD. Therefore, these results suggest not only the need for preventive management of individuals with low socioeconomic status but also that RD is a strong factor in the development of MACE.

**Conflicts of interest**

All authors have no conflicts of interest to declare.

**Funding**

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**Data sharing statement**

The data presented in this study are available on request from the corresponding author.

**Authors’ contributions**

Conceptualization: EHB, SYL, SWK  
Data curation: EHB, SYL, BK  
Formal analysis: TRO, BK, KDH  
Funding acquisition: HSC, SWK  
Methodology: EHB, TRO, BK, KDH  
Project administration, Resources: SWK  
Software: TRO, BK, KDH  
Supervision: SWK, KDH  
Validation: EMY, HSC, CSK, SKM  
Visualization: EMY, HSC, CSK  
Writing–original draft: EHB  
Writing–review & editing: SKM, SWK  
All authors read and approved the final manuscript.
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Introduction

Transurethral resection of the prostate (TURP) has become a gold standard for invasive endoscopic treatment of bladder outlet obstruction [1], i.e., benign prostatic hyperplasia (BPH) and prostate cancer. Although the procedure is relatively easy to perform, it entails a considerable risk of perioperative complications that may be acute (such as bleeding, bladder wall rupture or perforation) or chronic (such as incontinence and vesicoureteral and renal reflux) [2]. Dilutional (hypervolemic) hyponatremia remains one of the most prevalent acute complications of TURP. In rare...
but life-threatening situations it may lead to TURP hyponatremia syndrome [3]. The pathogenesis of post-TURP decreases in serum sodium has been well described, but routine risk assessment and prophylactic measures specific to the condition are still insufficient. The syndrome is caused by diffusion of hypoosmotic irritating fluid [3], most often tap water, into the vascular bed that leads to hemodilution, and thus a decrease in sodium concentration in the extracellular fluid. The development of hyponatremia induces homeostatic compensatory mechanisms including the inhibition of vasopressin excretion, which reduces reabsorption of water from the renal tubule, thereby increasing free water clearance.

The symptoms of TURP-related serum sodium decrease include nausea, confusion, bradycardia, increased blood pressure, visual disturbances, and, in severe cases, convulsions, pulmonary and brain edema, intussusception, and potentially death [4]. Most of the acute symptoms are the consequence of increased brain cell volume. In order to minimize the risk of TURP-related hyponatremia, the duration of surgery should be minimized, but other factors such as volume of instilled fluid and its composition must also be controlled [5]. However, such measures may not allow removal of the intended gland volume.

Several hormones might be risk markers for sodium disturbance after TURP, including atrial natriuretic peptide, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), and antidiuretic hormone (AVP). The assessment of AVP concentration in serum is, however, impeded by its short half-life period of only 5 to 20 minutes, and ex vivo and in vivo [6] instability, together with strong binding of the hormone to blood cells. These factors prevent the measurement of serum AVP concentration in routine diagnostics [6–8].

Copeptin (CPP) is a 39-amino acid peptide [9] produced and secreted with vasopressin in equimolar amounts and has good stability in circulation. Owing to these features, CPP is considered the best surrogate marker of vasopressin secretion in a range of human diseases related to its deficiency or access. CPP has been established as a reliable marker of inadequate response to hyponatremia treatment in patients with Schwartz–Bartter syndrome [10]. Another study investigated whether the determination of serum CPP together with brain natriuretic peptide (BNP) and prohormone of BNP in patients suffering from chronic heart failure complicated by hypervolemic hyponatremia could be feasible. An increase in serum CPP concentration was linked to increased mortality, regardless of clinical symptoms of the disease. CPP proved superior to BNP and NT-proBNP in this regard, although all these markers seemed to be closely associated [11]. Serum CPP may also predict survival in patients suffering from acute coronary syndrome, a condition that has been linked to increased vasopressin secretion [12,13]. In addition, assessment of CPP concentration in serum may predict the risk of outpatient mortality, irrespective of sodium concentration in plasma and dose of loop diuretics [14].

To the best of our knowledge, there have been no studies that investigated the potential utility of serum CPP as a marker of acute sodium disturbance following surgeries with a high risk of hyponatremia, including TURP. The aim of this study was to assess the role of serum CPP as a marker of the risk of hyponatremia in patients undergoing TURP.

Methods

The study protocol had been approved by the Ethics Committee of the Medical University of Lodz and written informed consent was obtained from each individual (No. RNN/90/13/KE from April 13, 2013). The study was performed in accordance with the Declaration of Helsinki for human studies. This study is registered at ClinicalTrials.gov (NCT03912766 11/Apr/2019).

Forty-nine patients were initially enrolled. Six enrolled patients were excluded from the final per-protocol analysis due to a lack of laboratory measurements after surgery. All patients underwent a TURP at a single regional urology center. The inclusion criteria were as follows: male sex, age of >45 years, glomerular filtration rate estimated from serum creatinine with the Chronic Kidney Disease Epidemiology Collaboration formula (eGFR) of >45 mL/min, diagnosis of BPH, and lower urinary tract symptoms. Exclusion criteria included acute infection, heart failure (New York Heart Association stage 3 or 4), diabetes insipidus, nephrogenic diabetes insipidus and other sodium homeostasis abnormalities, and/or impaired consciousness, psychogenic polydipsia or alcohol abuse. Patients receiving thiazide or loop diuretics, vasopressin or its analogues, steroids, and neuroleptics any time within 7 days before surgery were also excluded.
Surgery was performed under spinal anesthesia. The irrigation fluid container was placed 60 cm above the pubic symphysis. In order to minimize prostate volume estimation error, its size was estimated with ultrasound in three dimensions by the same technician using the same Samsung X8 (Samsung Medison) equipment in all patients. The duration of surgery was defined from the insertion of a resectoscope into the urinary bladder to the removal of endoscopic instruments from the urethra. The mass of prostate gland specimens was assessed immediately after excision in order to determine the total gland volume removed. For the purpose of the study, sodium and potassium concentration was determined directly before the start and 12 hours after surgery. Serum CPP concentration was determined before and 12 hours after the end of surgery. Serum NT-proBNP concentration was determined immediately before surgery. The aliquots were centrifuged in a refrigerated centrifuge and immediately frozen at –70 °C. Other lab tests included complete blood count, serum creatinine, and urinalysis with the determination of urine specific gravity and proteinuria.

Systolic and diastolic blood pressure and heart rate were measured 2 hours before and during the surgery 15, 30, and 60 minutes after its commencement, depending on the duration of the procedure, and then 30 minutes after its completion. The amount of fluids instilled intravenously was carefully measured during TURP. The fluids infused included 0.9% NaCl which contains 153 mmol/L sodium and Sterofundin (B. Braun) containing 140 mmol/L sodium. After surgery, each patient received 1,000 mL of 0.9% NaCl intravenously for 90 minutes. The volume of tap water used for flushing the bladder during surgery was assessed with an electronic flowmeter. The chemical composition of the tap water used during surgery was analyzed and it contained 0.28 mmol/L of sodium, 0.036 mmol/L of potassium, 24.6 mmol/L of calcium, and 0.074 mmol/L of magnesium. Serum and urine parameters were measured in the hospital laboratory with routine automated methods. Serum NT-proBNP was determined with electrochemiluminescence (ECLIA Roche Diagnostics) and serum CPP with the immunoenzymatic method (ELISA Cloud-Clone Corp.). The blood pressure before and after surgery was measured with a calibrated automatic oscillometric device (Omron M6; Omron). During surgery blood pressure was monitored with the monitor used for anesthesia.

The data are presented as an arithmetic mean ± standard deviation for normally distributed or median with interquartile range for non-normally distributed variables. The statistical significance of the within-group comparisons was assessed by paired t-test. The p-value of <0.05 was taken as significant. Correlations between the variables were calculated with the Pearson or Spearman parametric correlation coefficient depending on variable distribution. A multiple regression model was built with serum sodium change after TURP as a dependent variable. A receiver operator characteristic (ROC) curve was drawn using de Long’s method in order to determine the utility of biochemical parameters for serum sodium decrease risk estimation. The cut-off value ensuring the best sensitivity and specificity for each analyzed parameter was calculated using the Youden index. The statistical analysis was carried out with Statistica (version 13.1; TIBCO Software).

Sample size calculation was performed by using MedCalc (MedCalc Software). Power calculation showed that with a total number of 38 participants in two equal-sized subgroups, the analysis would have 80% power with significance of 0.05 for the comparison of the area under a ROC curve (AUC) with a null hypothesis value of 0.5 and expected AUC of 0.75.

**Results**

The baseline clinical and biochemical characteristics of the per-protocol study population are presented in Table 1. Table 2 shows the values of the parameters assessed at baseline and after surgery, as well as their absolute changes throughout the surgery and for 12 hours after TURP. The serum sodium concentration 12 hours after surgery was significantly lower than at baseline (p = 0.02). Although the absolute mean change in serum sodium levels during and after surgery was small, it was observed in 36 of 43 patients. Serum sodium decreased 12 hours after TURP to less than 130 mmol/L in only four patients. These patients (86, 85, 77, and 78 years old) were older than most of the study population (mean age of the whole group, 72.4 ± 9 years), but otherwise their clinical characteristics were similar to those who did not develop hyponatremia. These groups also did not differ significantly with respect to serum CPP and NT-proBNP. None of these patients had clinical signs of TURP syndrome. Serum CPP did not significantly increase.
Table 1. Baseline characteristics of the study group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>72.4 ± 9.0</td>
<td>73.0 (67.0–79.1)</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>86.5 ± 12.4</td>
<td>79.0 (75.0–87.0)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.5 ± 4.4</td>
<td>27.4 (26.5–28.7)</td>
</tr>
<tr>
<td>Mass of the removed prostate gland (g)</td>
<td>19.8 ± 16.3</td>
<td>15.0 (3.2–26.8)</td>
</tr>
<tr>
<td>Volume of the prostate gland (mL)</td>
<td>51.8 ± 34.0</td>
<td>40.0 (34.5–45.5)</td>
</tr>
<tr>
<td>Duration of surgery (min)</td>
<td>32.7 ± 13.4</td>
<td>30.0 (17.2–43.4)</td>
</tr>
<tr>
<td>Urine specific gravity, 1/1</td>
<td>1.01 ± 0.06</td>
<td>1.02 (1.002–1.032)</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>14.1 ± 1.7</td>
<td>14 (13.2–14.8)</td>
</tr>
<tr>
<td>Corpuscular volume (fl)</td>
<td>91.1 ± 4.2</td>
<td>91.0 (89.6–92.1)</td>
</tr>
<tr>
<td>Corpuscular Hb (pg)</td>
<td>31.1 ± 1.6</td>
<td>31.0 (30.5–32.2)</td>
</tr>
<tr>
<td>Corpuscular Hb concentration (g/dL)</td>
<td>34.0 ± 1.0</td>
<td>34.2 (33.6–35.0)</td>
</tr>
</tbody>
</table>

Hb, hemoglobin; IQR, interquartile range; SD, standard deviation.

from baseline to 12 hours after surgery (p = 0.34). Systolic blood pressure before surgery was significantly higher than after TURP (p < 0.001, respectively). Diastolic pressure before surgery was significantly higher (p = 0.03) than during the first 30 minutes of surgery and after surgery. Serum sodium before surgery correlated negatively with change in serum sodium for 12 hours from the start of surgery (r = -0.69, p < 0.001).

Serum CPP before surgery was negatively correlated with change in serum sodium for 12 hours from the start of surgery (R = -0.43, p = 0.004). No significant correlation was seen, however, between serum NT-proBNP concentration before surgery and change in serum sodium during and after surgery (p = 0.40).

A multiple regression model was built in order to analyze the effects of the variables that correlated linearly with the dependent variable on the variability of serum sodium concentration after surgery. Serum CPP before surgery and the duration of TURP explained most of the sodium concentration variation for 12 hours from the start of surgery (Table 3).

Fig. 1 shows the ROC curve with the decrease of serum sodium after surgery as a classification variable. The ROC analysis showed that serum CPP before surgery best predicted a decrease of serum sodium 12 hours after TURP (AUC, 0.775; 95% CI, 0.62–0.89; p < 0.001), with a cut-off point of >78.6 pg/mL, sensitivity of 77%, and specificity of 64.7%.

The plasma concentration of NT-proBNP before surgery did not predict serum sodium decrease 12 hours after TURP (AUC, 0.638; 95% CI, 0.48–0.78; p = 0.13) showing a cut-off point of ≤1,305, sensitivity of 100%, and specificity of 35.3%.

The total amount of bladder flushing fluid administered during surgery did not predict serum sodium decrease 12 hours after TURP (AUC, 0.602; 95% CI, 0.44–0.75; p = 0.24) showing a cut-off point ≤5.0, sensitivity of 38.5%, and specificity of 88.2%.

The total amount of fluid instilled intravenously during surgery predicted serum sodium decrease 12 hours after TURP with AUC of 0.0106 to 0.453 (p = 0.04). The AUC for the amount of sodium instilled intravenously was 0.543 (95% CI, 0.38–0.70), and that for serum CPP AUC was 0.775 (95% CI, 0.62–0.89).

Discussion

Despite the routine application of various measures that could prevent the development of hyponatremia during TURP such as maintaining the intrabladder pressure below 30 mmHg [15], use of continuous-flow resectoscopes, suprapubic drainage of the bladder [16], reduction of surgical duration to less than 1 hour, and leaving the tissue margins by the capsule of the gland where they can be left or excised completely, which reduces the duration of surgery [17,18], the risk of complication has not been fully eliminated and hyponatremia remains one of the most unwanted consequences of TURP surgery. Therefore, the estimation of the biochemical/hormonal markers that could allow the assessment of hyponatremia risk related to TURP remains feasible. That was why we decided to assess...
Table 2. Results of the assessments performed at baseline, during and after the surgery and the absolute changes of the parameters from baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline Mean ± SD</th>
<th>Median (IQR)</th>
<th>Total at the end of TURP Mean ± SD</th>
<th>Median (IQR)</th>
<th>After TURP Mean ± SD</th>
<th>Median (IQR)</th>
<th>Change before and after TURP Mean ± SD</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NT-proBNP (pg/mL)</td>
<td>928.0 ± 2,517.7</td>
<td>202.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Serum sodium (mmol/L)</td>
<td>141.0 ± 3.5</td>
<td>141.0</td>
<td>139.4 ± 3.3</td>
<td>139.0</td>
<td>1.6 ± 4.5e</td>
<td>–1.5</td>
<td>37.2 ± 248.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Serum CPP (pg/mL)</td>
<td>183.4 ± 221.2</td>
<td>85.4</td>
<td>223.4 ± 261.5</td>
<td>86.9</td>
<td>37.2 ± 248.7</td>
<td>4.1</td>
<td>(–2.8 to –0.1)</td>
<td>(–12.1 to 24.7)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.1 ± 0.3</td>
<td>1.0</td>
<td>125.0 ± 17.9f</td>
<td>120</td>
<td>NA</td>
<td>NA</td>
<td>(108.2–127.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Estimated GFR_{CKD-EPI} (mL/min)</td>
<td>74.4 ± 23.8</td>
<td>71.2</td>
<td>70.0 ± 9.4e</td>
<td>70.0</td>
<td>NA</td>
<td>NA</td>
<td>(65.2–73.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>134.5 ± 18.9</td>
<td>130.0</td>
<td>125.0 ± 17.9f</td>
<td>120</td>
<td>NA</td>
<td>NA</td>
<td>(108.2–127.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81.0 ± 10.5</td>
<td>80.0</td>
<td>70.0 ± 9.4e</td>
<td>70.0</td>
<td>NA</td>
<td>NA</td>
<td>(65.2–73.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Volume of IV fluids (mL)</td>
<td>NA</td>
<td>672.5 ± 338</td>
<td>600.0</td>
<td>600.0</td>
<td>NA</td>
<td>NA</td>
<td>(379.0–874.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Sodium content in IV fluids (mmol)</td>
<td>NA</td>
<td>102.4 ± 50.5</td>
<td>92.4</td>
<td>92.4</td>
<td>NA</td>
<td>NA</td>
<td>(48.1–131.1)</td>
<td>NA</td>
</tr>
<tr>
<td>Sodium content in IV fluids (mmol/kg body mass)</td>
<td>NA</td>
<td>1.3 ± 0.7</td>
<td>1.2</td>
<td>1.2</td>
<td>0.9–1.7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Intravesical fluid volume (L)</td>
<td>NA</td>
<td>7.7 ± 3.4</td>
<td>7.0</td>
<td>7.0</td>
<td>NA</td>
<td>NA</td>
<td>(5.8–8.8)</td>
<td>NA</td>
</tr>
<tr>
<td>Amount of sodium delivered to the bladder (mmol)</td>
<td>NA</td>
<td>2.1 ± 0.9</td>
<td>1.9</td>
<td>1.9</td>
<td>1.3–2.3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Amount of sodium delivered to the bladder (mmol/kg body mass)</td>
<td>NA</td>
<td>0.02 ± 0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>(0.01–0.03)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CPP, copeptin; GFR, glomerular filtration rate; IQR, interquartile range; IV, intravenous; NA, not applicable; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; SD, standard deviation; TURP, transurethral resection of prostate.

"Three hours before surgery," "15, 30, and 60 minutes after surgery," "12 hours after surgery; and " from 3 hours before to 12 hours after surgery." p = 0.02, † p < 0.001, ‡ p = 0.03.
the utility of CPP serum concentration estimated before surgery as a potential candidate marker for calculating the development risk of postoperative hyponatremia. The results of our study confirm that the assessment of the serum concentration of CPP before surgery might be useful to predict the development of TURP-related serum sodium decrease. We have also proved that the duration of surgery correlates significantly with serum sodium decrease after TURP, which means that surgery duration is the most important determinant of hyponatremia risk. In contrast, the estimation of other potential biomarkers of hyponatremia risk, such as presurgery serum NT-proBNP concentration, was found not to be predictive in our study. That was somewhat unexpected since serum BNP and NT-proBNP concentration is a well-recognized biomarker of heart failure and overhydration [11], and therefore we could expect that its measurement before surgery would improve the prediction of serum sodium decrease risk after TURP.

The study was comprised of a homogeneous group since we excluded the patients suffering from several chronic or acute diseases frequently leading to sodium disturbances and those taking medications that could influence the regulation of sodium balance. None of the patients suffered from infection which was particularly important since CPP is also a well-recognized marker of inflammation, including bacterial urinary tract infections [19]. The duration of surgery could not be standardized due to different anatomical conditions and ranged from 15 to 60 minutes (median, 32.7 minutes). According to the long-term practices of our and most other centers, tap water was used to irrigate the bladder in all patients. Tap water is used mostly due to its low cost and safety, which was confirmed in numerous studies [20]. However, the sodium concentration of tap water is much lower compared to plasma and may vary depending on location. Therefore we measured the sodium in the tap water that was used for TURP in our center. The low sodium content in tap water used for our study was only 2.81 mmol/L. In our study, mean serum sodium 12 hours after surgery was lower than before surgery. Similar changes were seen in other studies that investigated the utility of sodium concentration variations as a marker of TURP syndrome. In one of the studies, serum sodium concentration of >7.0 mmol/L and >7% was associated with the development of acute neurologic and cardiovascular symptoms [22]. The surgeons performing TURP also need to carefully monitor the pressure inside the bladder, which is a major factor affecting the absorption of water from irrigation fluid. Intrab-

**Figure 1.** ROC curves showing the predictive value of presurgery values of serum CPP and serum NT-proBNP, volumes of tap water used for bladder irrigation during surgery, and IV fluids infused during the TURP surgery for decreased serum sodium after surgery.

AUC, area under the curve; CI, confidence interval; CPP, copeptin; IV, intravenous; N-terminal prohormone of brain natriuretic peptide; ROC, receiver operator characteristic; TURP, transurethral resection of prostate.

**Table 3.** Regression coefficients for predicting change of serum sodium concentration for 12 hours from the beginning of transurethral resection of prostate procedure

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95% CI</th>
<th>β</th>
<th>t</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum copeptin before surgery</td>
<td>0.26</td>
<td>0.12–0.36</td>
<td>0.40</td>
<td>1.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Duration of the surgery</td>
<td>0.52</td>
<td>0.34–0.70</td>
<td>0.61</td>
<td>2.04</td>
<td>0.002</td>
</tr>
</tbody>
</table>

B, unstandardized β coefficient; β, standardized beta; CI, confidence interval; t, t-test statistics. 

$R^2_{\text{adj}} = 0.51$, $p = 0.02$, $F = 2.057$, degree of freedom = 6.36, standard error = 3.42.
ladder pressure should stay between 1 and 2.5 kPa in order to minimize the risk of dilutional hyponatremia during TURP [23]. In our study, the irrigation fluid was administered under a controlled pressure of 60 cmH\(_2\)O (5.88 kPa), with continuous drainage of water. For technical reasons, we could not monitor intrabladder pressure during surgery.

The volume of the prostate gland was measured with ultrasound with minimized inaccuracy since the measurement was always performed by the same technician.

The main limitation of our study was a small number of patients; however, the number was higher than the minimum required as estimated via power analysis. Our research should be treated as hypothesis generating; however, we tried to overcome this limitation by recruiting a homogenous group of patients undergoing TURP. Therefore, patients with cancer were excluded in accordance with the protocol. The limitation of the study was also the lack of pressure monitoring inside the bladder during surgery. Serum CPP was measured at only two time points.

In conclusion, serum CPP measured before surgery may be a marker for the risk of hyponatremia and TURP syndrome after TURP.

Conflicts of interest
All authors have no conflicts of interest to declare.

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Data sharing statement
The data presented in this study are available on request from the corresponding author.

Authors’ contributions
Conceptualization, Methodology: WB, MN
Investigation: WB, KBW
Formal analysis: MN
Writing-original draft: WB, KBW
Writing-review & editing: MN
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References


Association between urinary chloride excretion and progression of coronary artery calcification in patients with nondialysis chronic kidney disease: results from the KNOW-CKD study

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Background: Urine chloride has recently been suggested as a biomarker of renal tubule function in patients with nondialysis chronic kidney disease (CKD), as low urinary chloride concentration is associated with an increased risk of CKD progression. We investigate the association between urinary chloride excretion and the progression of coronary artery calcification (CAC).

Methods: A total of 1,065 patients with nondialysis CKD were divided into tertiles by spot urine chloride-to-creatinine ratios. The 1st, 2nd, and 3rd tertiles were defined as low, moderate, and high urinary chloride excretion, respectively. The study outcome was CAC progression, which was defined as an increase in coronary artery calcium score of more than 200 Agatston units during the 4-year follow-up period.

Results: Compared to moderate urinary chloride excretion, high urinary chloride excretion was associated with decreased risk of CAC progression (adjusted odds ratio, 0.379; 95% confidence interval, 0.190–0.757), whereas low urinary chloride excretion was not associated with risk of CAC progression. Restricted cubic spine depicted an inverted J-shaped curve, with a significant reduction in the risk of CAC progression in subjects with high spot urine chloride-to-creatinine ratios.

Conclusion: High urinary chloride excretion is associated with decreased risk of CAC progression in patients with nondialysis CKD.

Keywords: Biomarkers, Chronic renal insufficiency, Coronary artery disease, Urine chloride

Introduction

Chronic kidney disease (CKD) is a global health problem that imposes socio-economic burdens on the medical care system [1–3]. Risk stratification for cardiovascular (CV) disease in patients with CKD is an issue of particular
importance, as coronary artery disease (CAD), along with heart failure, is a leading cause of mortality and morbidity [4,5]. The risk of CAD is increased even in the early stages of CKD [6] and is further aggravated by the progression of CKD [7]. Because CKD staging is usually determined by the estimated glomerular filtration rate (eGFR) [8], the relationship between renal tubule function and the risk of CAD in this population is poorly understood.

Chloride is the most abundant anion in extracellular fluid and is delicately handled by specific channels and transporters [9,10]. The clinical investigation of urine chloride has been limited to the assessment of volume status [11] and calculation of anion gap [12], until a recent study reported associations of urinary chloride concentration with renal outcomes in patients with nondialysis CKD [13]. Based on the key finding that low urinary chloride concentration was significantly associated with an increased risk of CKD progression, the results suggested that adequate urinary chloride excretion may reflect functionally intact renal tubules [13]. Yet, to our best knowledge, the association between urinary chloride excretion and the risk of CAD has never been validated.

Assessments of coronary artery calcification (CAC) by cardiac computed tomography (CT) scans sensitively detect CAD, and have been validated for prediction of future CV events [14–17]. Taking advantage of the availability of cardiac CT images at baseline and 4-year follow-up for a sample of 1,065 patients with nondialysis CKD, we investigated the relationships between urinary chloride excretion and progression of CAC. We hypothesized that high urinary chloride excretion is associated with decreased risk of CAC progression. In addition, we conducted a series of subgroup analyses to determine whether the relationships between urinary chloride excretion and the risk of CAC progression are modified by clinical context.

Methods

Study design

The Korean Cohort Study for Outcomes in Patients With Chronic Kidney Disease (KNOW-CKD) is a nationwide prospective cohort study involving nine tertiary-care general hospitals in Korea (NCT01630486 at https://www.clinicaltrials.gov) [18]. Korean patients with CKD from stage 1 to predialysis stage 5 who voluntarily provided informed consent were enrolled from 2011 through 2016. The study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of participating centers, including Seoul National University Hospital (No. 1104-089-359), Yonsei University Severance Hospital (No. 4-2011-0163), Kangbuk Samsung Medical Center (2011-01-076), The Catholic University of Korea, Seoul St. Mary’s Hospital (No. KC1101OMI0441), Gil Hospital (No. GIRA2553), Eulji General Hospital (No. 201105-01), Chonnam National University Hospital (No. CNUH-2011-092), and Inje University Busan Paik Hospital (No. 11-091). All participants had been under close observation, and participants who experienced study outcomes were reported by each participating center. Among 2,238 participants who were longitudinally followed up, excluding those lacking baseline measurements of chloride and creatinine (Cr) in spot urine samples, and excluding those lacking either baseline or follow-up measurements of coronary artery calcium score (CACS), a total of 1,065 subjects were finally included in the analyses (Fig. 1).

Data collection from participants

Demographic information was collected from all eligible participants, including age, sex, comorbid conditions, primary renal disease, smoking history, and medication history (angiotensin-converting enzyme inhibitor [ACEi]/angiotensin II receptor blockers [ARBs], diuretics, number of anti-hypertension drugs, statins). Trained staff members measured the heights and weights of study participants. Body mass index (BMI) was calculated as weight divided by height squared. Systolic and diastolic blood pressures (SBP and DBP) were measured by an electronic sphygmomanometer after seated rest for 5 minutes. Venous samples were collected following overnight fasting, to determine hemoglobin, albumin, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), fasting glucose, high-sensitivity C-reactive protein (hsCRP), 25-hydroxyvitamin D (25(OH) vitamin D), sodium, potassium, chloride, and Cr levels at baseline. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation [19]. CKD stages were determined by the Kidney Disease Improving
Urinary chloride and CAC in CKD

Global Outcomes guidelines [8]. Urine albumin-to-Cr ratio (ACR) was measured in random, preferably second-voided, spot urine samples. Other urinary metrics, such as sodium, potassium, chloride, and Cr, were also measured in spot urine samples at baseline.

Measurement of coronary artery calcium score

Electrocardiography-gated coronary multidetector CT scans were checked following the standard protocol of each center at baseline and at year four follow-up visits. The CACS score was determined using Agatston units (AU) on a digital radiologic workstation [20].

Exposure and study outcome

The exposure of primary interest was urinary chloride-to-Cr ratio (Cl/Cr), which was used as a categorical variable. The subjects were divided into tertiles (T1, T2, and T3) by spot urine Cl/Cr. T1, T2, and T3 were defined as low, moderate, and high urinary chloride excretion, respectively. The study outcome was progression of CAC, which was defined as increase in CACS of more than 200 AU during the 4-year follow-up period, as in previously published studies from KNOW-CKD [21-23].

Statistical analysis

Continuous variables were expressed as the mean ± standard deviation or median (interquartile range). Categorical variables were expressed as the number of participants and percentage. Normality of distribution was ascertained by the Kolmogorov-Smirnov test. To compare baseline characteristics by urinary Cl/Cr, one-way analysis of variance and chi-square test were used for continuous and categorical variables, respectively. Univariate correlation analysis was performed with Spearman correlation analysis to assess the relationship between urine electrolyte parameters. Participants with any missing data in the primary analysis were excluded from further analyses. Binary logistic regression models were analyzed to address independent associations between urinary chloride excretion and the risk of CAC progression. The results of binary logistic regression models are presented as odds ratios (ORs) and 95%
confident intervals (CIs). Models were constructed after adjusting for the following variables. Model 1 represents crude ORs. Model 2 was adjusted for age, sex, Charlson comorbidity index, primary renal disease, current smoking status, medication (ACEi/ARBs, diuretics, number of antihypertensive drugs, statins), BMI, and SBP. Model 3 was further adjusted for hemoglobin, albumin, fasting glucose, HDL-C, TG, 25(OH) vitamin D, hsCRP, eGFR, and spot urine ACR. Model 4 was additionally adjusted for CACS at baseline. Restricted cubic splines were used to visualize the associations between urinary chloride excretion as a continuous variable and the OR for CAC progression. To validate our findings, we performed sensitivity analyses. First, participants with CACS of 0 AU at the baseline were excluded from binary logistic regression analysis, as CAC progression in those subjects was relatively rare. Second, we excluded subjects with eGFR of <15 mL/min/1.73 m², because there were relatively few subjects with eGFR of <15 mL/min/1.73 m², and including them may exaggerate the association between urinary chloride excretion and study outcomes due to advanced CKD. Third, we excluded subjects with eGFR of ≥90 mL/min/1.73 m², because those values are close to normal kidney function, and may not represent the CKD population well. Fourth, spot urine Na⁺/Cr and K⁺/Cr were included as covariates in binary logistic regression analysis. Lastly, we replaced the missing values in primary analyses by multiple imputations, and further conducted Cox regression analyses. To examine whether the association of urinary chloride excretion with the risk of CAC progression is modified by clinical contexts, we conducted prespecified subgroup analyses. Subgroups were defined by age (<60 years vs. ≥60 years), sex (male vs. female), BMI (<23 kg/m² vs. ≥23 kg/m²), eGFR (<45 mL/min/1.73 m² vs. ≥45 mL/min/1.73 m²), and spot urine ACR (<300 mg/gCr vs. ≥300 mg/gCr). Two-sided p-values of <0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS for Windows version 22.0 (IBM Corp.) and R version 4.1.1 (R project for Statistical Computing).

**Results**

**Baseline characteristics**

The baseline characteristics of the study participants are described by tertiles of spot urine Cl⁻/Cr (Table 1). BMI was highest in T3 and lowest in T1. The proportion of subjects using diuretics was highest in T1 and lowest in T3. The eGFR at baseline was highest in T3, and lowest in T1. Accordingly, the proportion of subjects in advanced stages of CKD was higher in T1. The other variables, including CACS at the baseline, did not show significant differences across groups. Correlation analysis to assess the relationships between urine electrolyte parameters revealed significant correlations among urinary sodium, potassium, and chloride excretion (Supplementary Table 1, available online).

**Association of spot urine Cl⁻/Cr and risk of coronary artery calcification progression in patients with nondialysis chronic kidney disease**

To determine independent associations of spot urine Cl⁻/Cr with the risk of CAC progression, we analyzed a binary logistic regression model (Table 2). Compared to moderate urinary chloride excretion, high urinary chloride excretion was associated with decreased risk of CAC progression (adjusted OR, 0.38; 95% CI, 0.19–0.76). Low urinary chloride excretion was not associated with either increased or decreased risk of CAC progression (adjusted OR, 0.72; 95% CI, 0.39–1.34). Restricted cubic spine depicted an inverted J-shaped curve, with a significant reduction of the risk for CAC progression in subjects with high spot urine Cl⁻/Cr (Fig. 2).

**Sensitivity analyses**

After excluding subjects with CACS of 0 AU at baseline, high urinary chloride excretion was still associated with decreased risk of CAC progression (adjusted OR, 0.36; 95% CI, 0.17–0.72) (Table 3). After excluding subjects at CKD stage 1, the association between high urinary chloride excretion with decreased risk of CAC progression was still significant (adjusted OR, 0.34; 95% CI, 0.16–0.74) (Supplementary Table 2, available online). After excluding subjects at CKD stage 5, the association between high urinary chloride excretion with decreased risk of CAC progression was still robust (adjusted OR, 0.37; 95% CI, 0.18–0.74) (Supplementary Table 3, available online). Even when spot urine Na⁺/Cr and K⁺/Cr were included in the regression model as covariates, the analysis demonstrated robust results (Supple-
Table 1. Baseline characteristics of study participants in the tertiles by spot urine Cl\(^{-}/Cr\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T1 (n = 355)</th>
<th>T2 (n = 355)</th>
<th>T3 (n = 355)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACS (AU)</td>
<td></td>
<td></td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>0</td>
<td>173 (48.7)</td>
<td>199 (56.1)</td>
<td>199 (56.1)</td>
<td></td>
</tr>
<tr>
<td>&gt;0, ≤400</td>
<td>151 (42.5)</td>
<td>133 (37.5)</td>
<td>136 (38.3)</td>
<td></td>
</tr>
<tr>
<td>&gt;400, ≤1,000</td>
<td>19 (5.4)</td>
<td>14 (3.9)</td>
<td>14 (3.9)</td>
<td></td>
</tr>
<tr>
<td>&gt;1,000</td>
<td>12 (3.4)</td>
<td>9 (2.5)</td>
<td>6 (1.7)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>52.57 ± 12.138</td>
<td>52.48 ± 11.985</td>
<td>52.82 ± 11.410</td>
<td>0.93</td>
</tr>
<tr>
<td>Male sex</td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td>Charlson comorbidity index</td>
<td></td>
<td></td>
<td></td>
<td>0.27</td>
</tr>
<tr>
<td>0–3</td>
<td>286 (80.3)</td>
<td>289 (81.4)</td>
<td>304 (85.6)</td>
<td></td>
</tr>
<tr>
<td>4–5</td>
<td>69 (19.4)</td>
<td>63 (17.7)</td>
<td>49 (13.8)</td>
<td></td>
</tr>
<tr>
<td>≥6</td>
<td>1 (0.3)</td>
<td>3 (0.8)</td>
<td>2 (0.6)</td>
<td></td>
</tr>
<tr>
<td>Primary renal disease</td>
<td></td>
<td></td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>60 (16.9)</td>
<td>54 (15.2)</td>
<td>53 (14.9)</td>
<td></td>
</tr>
<tr>
<td>HTN</td>
<td>74 (20.8)</td>
<td>76 (21.4)</td>
<td>70 (19.7)</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>131 (36.9)</td>
<td>115 (32.4)</td>
<td>145 (40.8)</td>
<td></td>
</tr>
<tr>
<td>TID</td>
<td>3 (0.8)</td>
<td>4 (1.1)</td>
<td>2 (0.6)</td>
<td></td>
</tr>
<tr>
<td>PKD</td>
<td>67 (18.9)</td>
<td>80 (22.5)</td>
<td>66 (18.6)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>26 (7.3)</td>
<td>20 (5.6)</td>
<td>19 (5.4)</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m(^2))</td>
<td>24.137 ± 3.294</td>
<td>24.394 ± 3.085</td>
<td>25.075 ± 3.342</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>125.465 ± 14.987</td>
<td>126.741 ± 15.104</td>
<td>125.600 ± 14.074</td>
<td>0.45</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.313 ± 10.903</td>
<td>77.220 ± 9.547</td>
<td>76.893 ± 10.421</td>
<td>0.494</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEi/ARBs</td>
<td>309 (87.0)</td>
<td>305 (85.9)</td>
<td>311 (87.6)</td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>100 (28.2)</td>
<td>86 (24.2)</td>
<td>71 (20.0)</td>
<td></td>
</tr>
<tr>
<td>No. of anti-HTN drugs ≥3</td>
<td>264 (74.4)</td>
<td>269 (75.8)</td>
<td>283 (79.7)</td>
<td>0.22</td>
</tr>
<tr>
<td>Statins</td>
<td>194 (56.6)</td>
<td>162 (45.6)</td>
<td>179 (50.4)</td>
<td>0.06</td>
</tr>
<tr>
<td>Laboratory finding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.226 ± 1.801</td>
<td>13.426 ± 1.852</td>
<td>13.488 ± 1.843</td>
<td>0.14</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.266 ± 0.338</td>
<td>4.249 ± 0.358</td>
<td>4.280 ± 0.325</td>
<td>0.47</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>173.285 ± 36.697</td>
<td>175.031 ± 35.631</td>
<td>175.652 ± 34.640</td>
<td>0.66</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>50.265 ± 15.685</td>
<td>51.733 ± 15.599</td>
<td>50.475 ± 14.254</td>
<td>0.38</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>96.101 ± 31.223</td>
<td>96.547 ± 29.204</td>
<td>98.756 ± 28.840</td>
<td>0.45</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>154.237 ± 93.455</td>
<td>151.877 ± 106.511</td>
<td>149.138 ± 82.377</td>
<td>0.78</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>105.283 ± 30.607</td>
<td>106.133 ± 28.387</td>
<td>107.756 ± 28.186</td>
<td>0.52</td>
</tr>
<tr>
<td>hsCRP (mg/dL)</td>
<td>0.600 (0.200–1.725)</td>
<td>0.600 (0.200–1.400)</td>
<td>0.600 (0.200–1.700)</td>
<td>0.54</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>140.932 ± 2.367</td>
<td>140.879 ± 2.273</td>
<td>141.048 ± 2.232</td>
<td>0.62</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>4.541 ± 0.500</td>
<td>4.461 ± 0.493</td>
<td>4.487 ± 0.480</td>
<td>0.099</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>104.783 ± 3.326</td>
<td>104.900 ± 3.027</td>
<td>105.006 ± 2.743</td>
<td>0.65</td>
</tr>
<tr>
<td>Spot urine Na(^+)/Cr (μmol/mgCr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K(^+)/Cr (μmol/mgCr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACR (mg/gCr)</td>
<td>64.820 ± 46.776</td>
<td>98.805 ± 47.067</td>
<td>142.452 ± 78.717</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m(^2))</td>
<td>54.263 ± 27.834</td>
<td>57.329 ± 28.410</td>
<td>66.943 ± 28.971</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CKD stage</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage 1</td>
<td>58 (16.3)</td>
<td>66 (18.6)</td>
<td>111 (31.3)</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>90 (25.4)</td>
<td>81 (22.8)</td>
<td>93 (26.2)</td>
<td></td>
</tr>
<tr>
<td>Stage 3a</td>
<td>58 (16.3)</td>
<td>83 (23.4)</td>
<td>64 (18.0)</td>
<td></td>
</tr>
<tr>
<td>Stage 3b</td>
<td>89 (25.1)</td>
<td>88 (24.8)</td>
<td>64 (18.0)</td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td>54 (15.2)</td>
<td>36 (10.1)</td>
<td>23 (6.5)</td>
<td></td>
</tr>
<tr>
<td>Stage 5</td>
<td>6 (1.7)</td>
<td>1 (0.3)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as number (%), mean ± standard deviation, or median (interquartile range).

ACEi, angiotensin-converting enzyme inhibitor; ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker; AU, Agatston unit; CACS, coronary artery calcium score; CKD, chronic kidney disease; Cl\(^{-}/Cr\), chloride-to-creatinine ratio; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; K\(^+\)/Cr, potassium-to-creatinine ratio; LDL-C, low-density lipoprotein cholesterol; Na\(^+\)/Cr, sodium-to-creatinine ratio; PKD, polycystic kidney disease; SBP, systolic blood pressure; T1, 1st tertile; T2, 2nd tertile; T3, 3rd tertile; TC, total cholesterol; TG, triglyceride; TID, tubulointerstitial disease.
Table 2. Binary logistic regression of spot urine Cl–/Cr for the risk of CAC progression

<table>
<thead>
<tr>
<th>Spot urine Cl–/Cr</th>
<th>Event, n (%)</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p-value</td>
<td>OR (95% CI)</td>
<td>p-value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>T1</td>
<td>46 (13.0)</td>
<td>0.98 (0.63–1.51)</td>
<td>0.91</td>
<td>0.97 (0.57–1.62)</td>
<td>0.89</td>
</tr>
<tr>
<td>T2</td>
<td>47 (13.2)</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>T3</td>
<td>25 (7.0)</td>
<td>0.50 (0.30–0.83)</td>
<td>0.007</td>
<td>0.45 (0.25–0.81)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Model 1: unadjusted model. Model 2: model 1 + adjusted for age, sex, Charlson comorbidity index, primary renal disease, current smoking status, medication (ACEi/ARBs, diuretics, number of anti-HTN drugs, statins), BMI, and SBP. Model 3: model 2 + adjusted for hemoglobin, albumin, fasting glucose, HDL-C, TG, 25(OH) vitamin D, hsCRP, eGFR, and spot urine ACR. Model 4: model 3 + adjusted for CACS at the baseline.

ACEi, angiotensin-converting enzyme inhibitor; ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker; BMI, body mass index; CAC, coronary artery calcification; CACS, coronary artery calcium score; CI, confidence interval; Cl–/Cr, chloride-to-creatinine ratio; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; OR, odds ratio; SBP, systolic blood pressure; T1, 1st tertile; T2, 2nd tertile; T3, 3rd tertile; TG, triglyceride.

Figure 2. Restricted cubic spline of spot urine Cl–/Cr on the risk of CAC progression. Adjusted OR of spot urine Cl–/Cr as a continuous variable for the progression of CAC is depicted. The model was adjusted for age, sex, Charlson comorbidity index, primary renal disease, current smoking status, medication (ACEi/ARBs, diuretics, number of anti-HTN drugs, statins), BMI, SBP, hemoglobin, albumin, fasting glucose, HDL-C, TG, 25(OH) vitamin D, hsCRP, eGFR, spot urine ACR, and CACS at the baseline.

ACEi, angiotensin-converting enzyme inhibitor; ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker; BMI, body mass index; CACS, coronary artery calcium score; Cl–/Cr, chloride-to-creatinine ratio; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; OR, odds ratio; SBP, systolic blood pressure; T1, 1st tertile; T2, 2nd tertile; T3, 3rd tertile; TG, triglyceride.

mentary Table 4, available online). Lastly, after replacing the missing values by multiple imputations, the association between high urinary chloride excretion with decreased risk of CAC progression remained robust (adjusted OR, 0.40; 95% CI, 0.20–0.78) (Supplementary Table 5, available online).
Table 3. Binary logistic regression of spot urine Cl/Cr for the risk of CAC progression in the subjects with baseline CACS of >0 AU

<table>
<thead>
<tr>
<th>Spot urine Cl/Cr</th>
<th>Event, n (%)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>46 (25.3)</td>
<td>0.81</td>
<td>0.39</td>
<td>0.79</td>
<td>0.41</td>
<td>0.73</td>
<td>0.29</td>
<td>0.64</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.50–1.31)</td>
<td></td>
<td>(0.46–1.38)</td>
<td></td>
<td>(0.40–1.31)</td>
<td></td>
<td>(0.34–1.22)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>46 (29.5)</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 (16.0)</td>
<td>1.04</td>
<td>&lt;0.001</td>
<td>0.79</td>
<td>0.005</td>
<td>0.39</td>
<td>0.004</td>
<td>0.36</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.02–1.07)</td>
<td></td>
<td>(0.46–1.38)</td>
<td></td>
<td>(0.20–0.74)</td>
<td></td>
<td>(0.17–0.72)</td>
<td></td>
</tr>
</tbody>
</table>

Model 1: unadjusted model. Model 2: model 1 + adjusted for age, sex, Charlson comorbidity index, primary renal disease, current smoking status, medication (ACEi/ARBs, diuretics, number of anti-HTN drugs, statins), BMI, and SBP. Model 3: model 2 + adjusted for hemoglobin, albumin, fasting glucose, HDL-C, TG, 25(OH) vitamin D, hsCRP, eGFR, and spot urine ACR. Model 4: model 3 + adjusted for CACS at the baseline.

Subgroup analyses

Subgroup analyses revealed that the association of urinary chloride excretion with the risk of CAC progression is not modified by age, sex, BMI, eGFR, or albuminuria (Table 4).

Discussion

In the present study, we demonstrated that high urinary chloride excretion is associated with decreased risk of CAC progression in patients with nondialysis CKD. The association depicted an inverted J-shaped relation, with a significant reduction of the risk for CAC progression in subjects with high spot urine Cl/Cr. Subgroup analyses revealed that the association is not modified by age, sex, BMI, eGFR, or albuminuria.

Urinary sodium and potassium excretion are correlated with dietary sodium and potassium intake, respectively [24,25]. As high urinary sodium or low urinary potassium excretion was associated with adverse CV outcomes in patients with CKD [25,26], it is interesting that the impact of urinary chloride excretion on the risk of CAC progression was not neutralized, considering that chloride ions are coupled with both sodium and potassium ions. This suggests that urinary chloride excretion does not simply estimate dietary chloride intake, and, rather, may represent the result of renal tubular handling of chloride ions that reflects distal delivery of chloride.

We speculate that adequate urinary excretion of chloride is a result of preserved renal function, thereby contributing to the prevention of CAC progression. A possible mechanism explaining how urinary chloride excretion guarantees a renoprotective effect is tubuloglomerular feedback (TGF) [27]. TGF is an autoregulatory mechanism that controls glomerular filtration rate (GFR). Increased distal flow, and corresponding increased distal delivery of chloride ions, is sensed by macula densa cells, leading to the release of adenosine from their basolateral sides, which in turn causes the constriction of glomerular afferent arterioles [27–29]. This collectively prevents unopposed increases in GFR, and assists in the maintenance of intratubular flow rate. From the perspective of pathophysiology, the activation of TGF functions as a defensive mechanism against glomerular hypertension, which has been shown to be effective in the long-term preservation of GFR [30–33]. Although increased delivery of sodium ions may also initiate TGF response, animal studies demonstrated the predominant role of chloride, rather than sodium, ions as stimulation for macula densa cells to drive TGF [28,34]. It is assumed that, therefore, adequate activation of TGF response is mirrored in high urinary chloride excretion, ultimately contributing to the preservation of kidney function, and to the prevention of CAC prevention. We speculate that the predictive value of urinary chloride excretion as a biomarker of CAC progression is substantial, because despite there being no association between urinary chloride excretion and CACS at baseline, the risk of CAC progression significantly differed by baseline urinary chloride excretion in 4 years.

It is well-known that the risk of CAD is increased even in the early stages of CKD [6], while the risk is further aggravated by progression of CKD [7], which is usually determined by eGFR [8]. Although urinary chloride excretion is
postulated to be a biomarker of renal tubule function, we found that it was also correlated with eGFR (Table 1), suggesting that the function of the renal tubule is closely related to that of the glomerulus. Thus, it seems reasonable that urinary chloride excretion predicts the risk of CAC progression. Nevertheless, it is still intriguing that the regression models in the current study include eGFR as a co-variable, which means that the association of urinary chloride ex-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Spot urine Cl⁻/Cr</th>
<th>Unadjusted</th>
<th>p for interaction</th>
<th>Adjusted</th>
<th>p for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p</td>
<td>OR (95% CI)</td>
<td>p</td>
<td></td>
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<tr>
<td>Age (yr)</td>
<td></td>
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<tr>
<td>&lt;60</td>
<td>T1 0.62 (0.31–1.20)</td>
<td>0.09</td>
<td>0.31 (0.08–1.08)</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td></td>
<td>T3 0.28 (0.11–0.63)</td>
<td>0.14</td>
<td>0.14 (0.02–0.70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60 yr</td>
<td>T1 1.50 (0.81–2.79)</td>
<td></td>
<td>0.80 (0.33–1.90)</td>
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<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td></td>
<td>T3 0.74 (0.37–1.46)</td>
<td></td>
<td>0.42 (0.16–1.06)</td>
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<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>T1 0.89 (0.53–1.49)</td>
<td>0.58</td>
<td>0.61 (0.29–1.25)</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
<td></td>
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<tr>
<td></td>
<td>T3 0.54 (0.30–0.97)</td>
<td></td>
<td>0.38 (0.16–0.84)</td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>T1 1.22 (0.53–2.87)</td>
<td></td>
<td>2.03 (0.19–29.48)</td>
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</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td></td>
<td>T3 0.40 (0.12–1.13)</td>
<td></td>
<td>0.01 (0.00–0.27)</td>
<td></td>
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<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;23</td>
<td>T1 1.17 (0.48–2.93)</td>
<td>0.93</td>
<td>0.62 (0.10–4.04)</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3 0.51 (0.14–1.64)</td>
<td></td>
<td>0.23 (0.01–2.68)</td>
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</tr>
<tr>
<td>≥23</td>
<td>T1 0.96 (0.57–1.58)</td>
<td></td>
<td>0.65 (0.30–1.37)</td>
<td></td>
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<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
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</tr>
<tr>
<td></td>
<td>T3 0.47 (0.26–0.81)</td>
<td></td>
<td>0.37 (0.16–0.79)</td>
<td></td>
<td></td>
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<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>≥45</td>
<td>T1 0.53 (0.26–1.02)</td>
<td>0.07</td>
<td>0.49 (0.18–1.22)</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>T3 0.42 (0.21–0.79)</td>
<td></td>
<td>0.28 (0.11–0.70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>T1 1.54 (0.84–2.91)</td>
<td></td>
<td>1.41 (0.54–3.78)</td>
<td></td>
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<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
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<tr>
<td></td>
<td>T3 0.71 (0.30–1.57)</td>
<td></td>
<td>0.38 (0.10–1.29)</td>
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</tr>
<tr>
<td>Spot urine ACR (mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;300</td>
<td>T1 0.92 (0.47–1.80)</td>
<td>0.86</td>
<td>0.45 (0.15–1.28)</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3 0.56 (0.26–1.16)</td>
<td></td>
<td>0.34 (0.11–1.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥300</td>
<td>T1 0.99 (0.56–1.79)</td>
<td></td>
<td>0.90 (0.37–2.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2 Reference</td>
<td></td>
<td>Reference</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>T3 0.45 (0.22–0.90)</td>
<td></td>
<td>0.28 (0.08–0.88)</td>
<td></td>
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</tr>
</tbody>
</table>

The model was adjusted for age, sex, Charlson comorbidity index, primary renal disease, current smoking status, medication (ACEi/ARBs, diuretics, number of anti-HTN drugs, statins), BMI, SBP, hemoglobin, albumin, fasting glucose, HDL-C, TG, 25(OH) vitamin D, hsCRP, eGFR, spot urine ACR, and CACS at the baseline.

ACEi, angiotensin-converting enzyme inhibitor; ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker; BMI, body mass index; CAC, coronary artery calcification; CACS, coronary artery calcium score; CI, confidence interval; Cl⁻/Cr, chloride-to-creatinine ratio; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; OR, odds ratio; SBP, systolic blood pressure; T1, 1st tertile; T2, 2nd tertile; T3, 3rd tertile; TG, triglyceride.
cretion with the risk of CAC progression is independent of eGFR. It should be, therefore, further clarified whether the primary defect in renal tubular handling of chloride excretion may increase the risk of CAC progression.

Several limitations are to be acknowledged in the current study. First, we cannot determine a causal relation between urinary chloride excretion and the risk of CAC progression, because of the observational nature of the current study. Second, despite the robust findings for the association between urinary chloride excretion and the risk of CAC progression, we were not able to identify the precise mechanism underlying the association. Third, we did not examine whether urinary chloride excretion is also associated with the overall CV outcomes in patients with nondialysis CKD. Fourth, as this cohort study enrolled only ethnic Koreans, precautions are required to extrapolate the data to other populations.

In conclusion, we report that high urinary chloride excretion is associated with decreased risk of CAC progression in patients with nondialysis CKD. Further studies are warranted to unveil the precise mechanism underlying the association between urinary chloride excretion and the risk of CAC progression and to determine whether urinary chloride excretion is also associated with overall CV outcomes in patients with nondialysis CKD.

Conflicts of interest

Tae-Hyun Yoo is the Editor-in-Chief of Kidney Research and Clinical Practice and was not involved in the review process of this article. All authors have no other conflicts of interest to declare.

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Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors’ contributions

Conceptualization, Methodology: SHS
Data curation, Formal analysis: SHS, TRO, HSC
Investigation: CSK, EHB, SKM
Supervision: KHO, THY, DWC, SWK
Writing–original draft: SHS
Writing–review & editing: SHS, SWK
All authors read and approved the final manuscript.

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References


Serum calcification propensity and its association with biochemical parameters and bone mineral density in hemodialysis patients

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2 Division of Nephrology, Department of Internal Medicine, Gachon University Gil Medical Center, Gachon University College of Medicine, Incheon, Republic of Korea

Background: T50 is a novel serum-based marker that assesses the propensity for calcification in serum. A shorter T50 indicates a greater propensity to calcify and has been associated with cardiovascular disease and mortality among patients with chronic kidney disease. The factors associated with T50 and the correlation between T50 and bone mineral density (BMD) are unknown in hemodialysis (HD) patients.

Methods: This cross-sectional study included 184 patients undergoing HD. Individuals were grouped into tertiles of T50 to compare the demographic and disease indicators of the tertiles. Linear regression was used to evaluate the association between T50 and hip and spinal BMD in a multivariate model.

Results: Mineral and inflammatory parameters, including serum phosphate (r = −0.156, p = 0.04), albumin (r = 0.289, p < 0.001), and high-sensitivity C-reactive protein (r = −0.224, p = 0.003) levels, were associated with T50. We found a weak association between T50 and BMD in the total hip area in the unadjusted model (β = 0.030, p = 0.04) but did not find a statistically significant association with the total hip (β = 0.017, p = 0.12), femoral neck (β = −0.001, p = 0.96), or spinal BMD (β = 0.019, p = 0.33) in multivariable-adjusted models.

Conclusion: T50 was moderately associated with mineral and inflammatory parameters but did not conclusively establish an association with BMD in HD patients. Broad-scale future studies should determine whether T50 can provide insights into BMD beyond traditional risk factors in this population.

Keywords: Bone mineral density, Hemodialysis, T50, Vascular calcification

Introduction

Chronic kidney disease (CKD)-mineral bone disease (MBD) is a common complication of CKD that is associated with morbidity and mortality. Several studies have suggested an interconnection between vascular calcification, impaired bone and mineral metabolism, and increased mortality [1–3]. Recent studies have found that bone mineral density (BMD) measurement in patients with advanced CKD predicts the risk of fracture, which can be expected to provide...
nephrologists with skeletal fragility and targeted fracture prevention strategies [4–6].

T_{50} has been proposed as a potential novel serum-based marker for assessing calcification propensity [7]. With the initiation of calcium and phosphate precipitation in the serum, primary calciprotein particles (CPP) are formed that are rich in calcium and phosphate and contain small amounts of protein, including albumin and fetuin-A [7]. Over time, these primary CPPs are converted into larger secondary CPPs with different calcium, phosphate, and protein content. CPP maturation time (T_{50}) is a measurement of the in vitro conversion time from primary CPP to secondary CPP in the serum [7]. The balance of calcification enhancing and inhibitory factors in each serum sample is a critical factor in determining transformation time [8]. The shorter the T_{50}, the greater the tendency for calcification. A shorter T_{50} has been reported to be associated with increased risk of cardiovascular disease (CVD) and all-cause mortality in CKD patients [8,9].

Although vascular calcification and bone health are intercorrelated and are known risk factors for predicting cardiovascular events (CVE) in dialysis patients, the association between T_{50} and BMD in dialysis patients with a high CVE risk is not well understood. As renal function decreases in CKD patients, mineral parameters are perturbed and related to bone and vascular health, which is an important pathophysiology of CKD-MBD [10]. T_{50} also tends to accompany mineral parameters in serum in the CKD environment, indicating the tendency for vascular calcification [11].

In the past, routine BMD evaluation was not recommended in CKD patients [12], but fractures can be predicted by measuring BMD in non-dialysis-dependent CKD (CKD-ND) patients [13] and end-stage kidney disease (ESKD) patients on hemodialysis (HD) [6]. In addition, as osteoporosis treatments for patients with impaired renal function have been developed, BMD measurement is being actively performed. However, in the pathophysiology of CKD-MBD, it is difficult to reflect bone quality because the bone density of the trabecular bone may be overestimated [12,14]. A recent study reported that CKD showed a correlation with low BMD measured at the hip, but not with BMD measured at the spine [13]. Until now, osteopenia and osteoporosis have been diagnosed using the same cut-off values as in the general population [14], but follow-up studies on bone health are needed in ESKD patients.

Therefore, in this study, we aimed to provide the first analysis of the clinical and biochemical parameters of T_{50} in patients undergoing HD. We also examined the relationships between T_{50}, BMD from the various sites, and mineral and inflammatory parameters, to evaluate the potential of T_{50} as a predictor of the CKD-MBD association in HD patients.

**Methods**

**Study design and setting**

This study was based on maintenance HD patients from a single center in Korea. We investigated the associations between T_{50}, BMD, and biochemical parameters using a cross-sectional design.

**Study population**

A total of 184 patients who visited our HD unit at the Gachon University Gil Medical Center between March 2020 and February 2021 were analyzed. Patients were enrolled in the study if they 1) had been on HD for at least 3 months, 2) agreed to participate in the study with written informed consent, and 3) were free of any complications that could affect serum T_{50} and other biochemical parameters such as an indwelling catheter, any underlying malignancy, active liver disease, current infection, or previous parathyroidectomy.

This study adheres to the Declaration of Helsinki and was approved by the Institutional Review Board at the Gachon University Gil Medical Center (No. GBIRB2020-342). Written informed consent was obtained from all participants.

**Clinical and laboratory parameters**

All demographic and clinical data, comorbidities, laboratory values, and medications were collected at the time of enrollment from participants’ medical records by a well-trained study coordinator. The following baseline demographic and clinical characteristics were collected: age, sex, body mass index, smoking, and HD duration. Data on comorbidities, including hypertension (HTN), diabetes mellitus (DM), CVD such as angina pectoris, myocardial infarction, heart failure (HF), transient ischemic attack (TIA), stroke, and peripheral arterial disease, were also collected. Angina pectoris and myocardial infarction were defined as...
the presence of coronary artery disease as documented by angiography, an acute coronary syndrome, angina requiring percutaneous coronary intervention, or coronary artery bypass grafting surgery. Stroke and TIA were defined as cases where magnetic resonance imaging was performed on patients with suspected symptoms that were diagnosed by a neurologist. Systolic HF was defined as left ventricular ejection fraction of <40%, and diastolic HF was defined as E/é of >15. All blood samples were obtained prior to a mid-week HD session after overnight fasting and microcentrifugation for measurements. Serum was separated from blood samples within 1 hour of collection and stored at −70 °C until analysis. Laboratory data included the single-pool Kt/V (spKt/V), hemoglobin, albumin, protein, calcium, phosphorus, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, parathyroid hormone, alkaline phosphatase (ALP), total cholesterol, triglyceride (TG), and high-sensitivity C-reactive protein (hsCRP). Medication data included the use of renin-angiotensin-aldosterone system blockers calcium channel blockers, β-blockers, phosphate binders, statin, vitamin D analogues, and cinacalcet.

**Determination of the serum calcification propensity (T\textsubscript{50})**

T\textsubscript{50} was determined using a nephelometer (Nephelostar; BMG Labtech, Offenburg, Germany), which measures the time-point transformation from primary to secondary CPP, as described in a previous study [7]. To this end, patient serum (80 μL) was first exposed to NaCl solution (20 μL), followed by high and supersaturated concentrations of calcium (50 μL) and phosphate (50 μL) solutions. The experiment was performed in triplicate in a 96-well plate. The Nephelostar was operated and controlled using Galaxy software. Nonlinear regression curves were calculated for the determination of T\textsubscript{50}. The analytical coefficients of variation of standards precipitated at 120, 240, and 360 minutes were 9.8%, 8.7%, and 8.4%, respectively.

**Measurement of bone mineral density and abdominal aortic calcification score**

The BMD was estimated using a dual-energy X-ray absorptiometry system (Hologic, Marlborough, MA, USA). The BMD of the total hip, femoral neck, and lumbar spine (L1–L4) were measured at baseline, and the results were expressed as density (g/cm\textsuperscript{2}) and T-scores (standard deviation [SD] from the average BMD value in a healthy young population).

Plain X-ray images of the lateral lumbar spine from all subjects were studied to calculate semiquantitative abdominal aortic calcification (AAC) scores, as described by Kauppila et al. [15]. The AAC score was graded on a 0 to 3 scale at each segment (L1–L4) of the lumbar vertebrae based on the severity of calcification as follows: 0, no calcific deposits; 1, small scattered calcific deposits less than 1/3 of the longitudinal wall of the aorta; 2, 1/3 or more but less than 2/3; and 3, 2/3 or more. The anterior and posterior wall scores were separately graded and summed, resulting in a total score of 0 to 24. All X-ray images were analyzed by two independent observers having no knowledge of the clinical history of each subject, and consensus was reached on the interpretation of all radiographs.

**Statistical analyses**

Continuous variables were tested for normality using the Shapiro-Wilk test before further statistical analysis. Variables without a normal distribution were either transformed into a logarithmic scale and then subjected to parametric tests or analyzed using a non-parametric test. Values with a normal distribution are expressed as mean ± SD, while those without a normal distribution are presented as median and interquartile range. Comparisons between the groups were performed using the chi-square test, Student t test, or analysis of variance with Tukey multiple comparison test as appropriate. Correlation between two continuous variables was analyzed using Pearson correlation test. Variables that do not show a normal distribution were analyzed by converting them to logarithmic values. Independent variables associated with T\textsubscript{50} were identified using multiple stepwise linear regression analysis. All statistical analyses were conducted using R software, version 3.5.3 with packages (The Comprehensive R Archive Network; http://cran.r-project.org). For all statistical analyses, statistical significance was set at p < 0.05.

**Results**

**Characteristics of the study population**

Participant demographics and clinical characteristics strat-
ified by tertiles of $T_{50}$ concentration are shown in Table 1. The mean $T_{50}$ was 296 ± 85 minutes. Ninety-six participants (52.2%) were men, mean age was 61 ± 12 years, mean dialysis duration was approximately 107 months, and there was a high prevalence of comorbidities such as DM (47.3%), HTN (58.7%), and previous CVD (40.8%). Descending tertiles of serum $T_{50}$ were associated with lower serum albumin (3.9 ± 0.4, 4.0 ± 0.3, 4.1 ± 0.3; p < 0.001) and TG (85.4 ± 68.8, 106.2 ± 74.6, 113.6 ± 72.8; p = 0.03) levels as well as higher serum hsCRP (0.2 [0.1–0.5], 0.2 [0.0–0.3], 0.1 [0.0–0.3]; p = 0.03), phosphate (5.6 ± 1.8, 5.3 ± 1.3, 5.0 ± 1.0; p = 0.02), and ALP (125.4 ± 95.7, 98.3 ± 41.2, 102.0 ± 37.5; p = 0.045) concentrations (Table 1).

Table 1. Baseline characteristics of the study group according to tertiles of serum $T_{50}$

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>184</td>
<td>61</td>
<td>62</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>$T_{50}$ (min)</td>
<td>296.3 ± 85.3</td>
<td>204.1 ± 39.0</td>
<td>290.9 ± 25.3</td>
<td>394.1 ± 40.3</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>61.1 ± 12.3</td>
<td>61.8 ± 12.5</td>
<td>58.7 ± 13.1</td>
<td>62.8 ± 10.9</td>
<td>0.65</td>
</tr>
<tr>
<td>Male sex</td>
<td>96 (52.2)</td>
<td>33 (54.1)</td>
<td>32 (51.6)</td>
<td>31 (50.8)</td>
<td>0.93</td>
</tr>
<tr>
<td>HD duration (mo)</td>
<td>107 (64–139)</td>
<td>120 (71–147)</td>
<td>92 (69–139)</td>
<td>104 (52–127)</td>
<td>0.10</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>23.5 ± 3.8</td>
<td>23.1 ± 3.9</td>
<td>23.8 ± 3.8</td>
<td>23.5 ± 3.8</td>
<td>0.56</td>
</tr>
<tr>
<td>Smoking</td>
<td>28 (15.2)</td>
<td>10 (16.4)</td>
<td>9 (14.5)</td>
<td>9 (14.8)</td>
<td>0.95</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>87 (47.3)</td>
<td>25 (41.0)</td>
<td>29 (46.8)</td>
<td>33 (54.1)</td>
<td>0.35</td>
</tr>
<tr>
<td>Hypertension</td>
<td>108 (58.7)</td>
<td>36 (59.0)</td>
<td>37 (59.7)</td>
<td>35 (57.4)</td>
<td>0.97</td>
</tr>
<tr>
<td>CVD</td>
<td>75 (40.8)</td>
<td>27 (44.3)</td>
<td>21 (33.9)</td>
<td>27 (44.3)</td>
<td>0.40</td>
</tr>
<tr>
<td>spKtV</td>
<td>1.6 (1.4–1.8)</td>
<td>1.6 (1.4–1.9)</td>
<td>1.6 (1.4–1.8)</td>
<td>1.6 (1.4–1.8)</td>
<td>0.96</td>
</tr>
<tr>
<td>RAS blockade</td>
<td>79 (42.9)</td>
<td>26 (42.6)</td>
<td>28 (45.2)</td>
<td>25 (41.0)</td>
<td>0.90</td>
</tr>
<tr>
<td>CCB</td>
<td>81 (44.0)</td>
<td>29 (47.5)</td>
<td>28 (45.2)</td>
<td>23 (37.7)</td>
<td>0.64</td>
</tr>
<tr>
<td>β-blocker</td>
<td>80 (43.5)</td>
<td>29 (47.5)</td>
<td>28 (45.2)</td>
<td>23 (37.7)</td>
<td>0.52</td>
</tr>
<tr>
<td>Phosphate binder</td>
<td>131 (71.2)</td>
<td>44 (72.1)</td>
<td>42 (67.7)</td>
<td>45 (73.8)</td>
<td>0.75</td>
</tr>
<tr>
<td>Statin</td>
<td>71 (38.6)</td>
<td>24 (39.3)</td>
<td>22 (35.5)</td>
<td>25 (41.0)</td>
<td>0.81</td>
</tr>
<tr>
<td>Vitamin D analogues</td>
<td>123 (66.8)</td>
<td>41 (67.2)</td>
<td>37 (59.7)</td>
<td>45 (73.8)</td>
<td>0.25</td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>17 (9.2)</td>
<td>8 (13.1)</td>
<td>5 (8.1)</td>
<td>4 (6.6)</td>
<td>0.42</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>10.8 ± 1.3</td>
<td>10.7 ± 1.3</td>
<td>10.7 ± 1.2</td>
<td>11.1 ± 1.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.0 ± 0.3</td>
<td>3.9 ± 0.4</td>
<td>4.0 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>137.0 ± 34.5</td>
<td>128.7 ± 28.6</td>
<td>141.9 ± 37.6</td>
<td>140.2 ± 35.6</td>
<td>0.07</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>101.9 ± 72.7</td>
<td>85.4 ± 68.8</td>
<td>106.2 ± 74.6</td>
<td>113.6 ± 72.8</td>
<td>0.03</td>
</tr>
<tr>
<td>hsCRP (mg/dL)</td>
<td>0.1 (0.0–0.4)</td>
<td>0.2 (0.1–0.5)</td>
<td>0.2 (0.0–0.3)</td>
<td>0.1 (0.0–0.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.2 ± 0.9</td>
<td>8.3 ± 0.9</td>
<td>8.2 ± 1.0</td>
<td>8.2 ± 0.9</td>
<td>0.70</td>
</tr>
<tr>
<td>Phosphate (mg/dL)</td>
<td>5.3 ± 1.4</td>
<td>5.6 ± 1.8</td>
<td>5.3 ± 1.3</td>
<td>5.0 ± 1.0</td>
<td>0.02</td>
</tr>
<tr>
<td>VD₃ (ng/mL)</td>
<td>17.2 ± 9.8</td>
<td>17.4 ± 10.4</td>
<td>17.9 ± 10.1</td>
<td>16.4 ± 8.9</td>
<td>0.61</td>
</tr>
<tr>
<td>VD₁₂₅ (pg/mL)</td>
<td>5.9 ± 7.1</td>
<td>5.7 ± 7.4</td>
<td>5.7 ± 7.0</td>
<td>6.4 ± 7.1</td>
<td>0.61</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>564.2 ± 380.9</td>
<td>649.1 ± 501.2</td>
<td>504.2 ± 293.5</td>
<td>541.7 ± 306.9</td>
<td>0.12</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>108.5 ± 64.6</td>
<td>125.4 ± 95.7</td>
<td>98.3 ± 41.2</td>
<td>102.0 ± 37.5</td>
<td>0.045</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>4.0 (0.0–12.0)</td>
<td>4.0 (0.0–10.5)</td>
<td>3.5 (0.0–8.0)</td>
<td>5.0 (0.0–11.0)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Data are expressed as number only, mean ± standard deviation, number (%), or median (interquartile range).

AAC, abdominal aortic calcification; ALP, alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; CCB, calcium channel blocker; CVD, cardiovascular disease; HD, hemodialysis; hsCRP, highly selective C-reactive protein; PTH, parathyroid hormone; RAS, renin-angiotensin-aldosterone; spKtV, single-pool Kt/V; T1, 1st tertile; T2, 2nd tertile; T3, 3rd tertile; VD₁₂₅, 1,25-dihydroxyvitamin D; VD₂₅, 25-hydroxyvitamin D.
Correlation between serum T\textsubscript{50} and related parameters

T\textsubscript{50} showed a significant correlation with the total hip T-score (r = 0.158, p = 0.038) (Table 2). However, there was no significant correlation between the T\textsubscript{50} and AAC scores on plain radiographs (r = 0.064, p = 0.401) (Table 2). With respect to medication use, there was no significant correlation between the descending tertiles of serum T\textsubscript{50} and medications for CKD-MBD, including phosphate binders, vitamin D analogues, and cinacalcet. Serum T\textsubscript{50} was positively correlated with serum albumin concentration (r = 0.289, p < 0.001) (Fig. 1). In addition, it was inversely correlated with serum hsCRP (r = –0.224, p = 0.003) and phosphate (r = –0.156, p = 0.040) concentrations (Fig. 1).

Association between bone mineral density and related parameters

We compared the mean T-score of BMD according to the sites at which it was assessed (Fig. 2). The mean T-score for BMD measured at the femur neck was relatively lower than that for the BMD assessed at the total hip or lumbar spine (–1.9 ± 1.2, –1.6 ± 1.3, and –1.1 ± 1.8, respectively). There was a significant difference between the three groups (p < 0.001). In the multiple comparison test by Tukey method, there were also significant differences between femur neck and L spine (p < 0.001) and between total hip and L spine (p = 0.001), but the difference between femur neck and total hip was not significant (p = 0.26).

BMD showed an inverse correlation with age and the spKtV (lumbar: r = –0.310, p < 0.001; femoral neck: r = –0.403, p < 0.001; total hip: r = –0.440, p < 0.001) and a positive correlation with albumin (lumbar: r = 0.094, p = 0.218; femoral neck: r = 0.201, p = 0.008; total hip: r = 0.219, p = 0.004). Only the L spine BMD showed an inverse correlation with ALP (r = –0.225, p = 0.003). Femoral neck (r = –0.267, p < 0.001) and total hip BMD (r = –0.176, p = 0.021) both showed an inverse relationship with AAC scores (Table 2).

Table 2. Cross-sectional correlation analyses between serum T\textsubscript{50} and BMD and other variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Age</th>
<th>spKtV</th>
<th>Albumin</th>
<th>hsCRP*</th>
<th>Calcium</th>
<th>Phosphate</th>
<th>PTH</th>
<th>ALP</th>
<th>BMD_LS</th>
<th>BMD_FN</th>
<th>BMD_TH</th>
<th>AAC*</th>
<th>T\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spKtV</td>
<td>0.226*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>–0.245*</td>
<td>–0.015</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP*</td>
<td>0.025</td>
<td>–0.120</td>
<td>–0.205*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>0.020</td>
<td>0.037</td>
<td>0.242*</td>
<td>–0.107</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>–0.350*</td>
<td>–0.213*</td>
<td>0.191*</td>
<td>0.097</td>
<td>0.063</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>–0.193*</td>
<td>–0.215*</td>
<td>0.113</td>
<td>0.010</td>
<td>0.193*</td>
<td>0.342*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>0.089</td>
<td>–0.029</td>
<td>0.058</td>
<td>0.011</td>
<td>0.085</td>
<td>–0.032</td>
<td>0.339*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD_LS</td>
<td>–0.180*</td>
<td>–0.310*</td>
<td>0.094</td>
<td>0.056</td>
<td>–0.026</td>
<td>0.105</td>
<td>–0.069</td>
<td>–0.225*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD_FN</td>
<td>–0.513*</td>
<td>–0.403*</td>
<td>0.201*</td>
<td>–0.003</td>
<td>0.028</td>
<td>0.132</td>
<td>0.011</td>
<td>–0.019</td>
<td>0.599*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD_TH</td>
<td>–0.353*</td>
<td>–0.440*</td>
<td>0.219*</td>
<td>0.053</td>
<td>0.044</td>
<td>0.061</td>
<td>–0.005</td>
<td>–0.103</td>
<td>0.599*</td>
<td>0.800*</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC*</td>
<td>0.443*</td>
<td>–0.009</td>
<td>–0.126</td>
<td>0.089</td>
<td>0.063</td>
<td>–0.051</td>
<td>–0.072</td>
<td>0.126</td>
<td>0.024</td>
<td>–0.267*</td>
<td>–0.176*</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>T\textsubscript{50}</td>
<td>0.042</td>
<td>–0.006</td>
<td>0.289*</td>
<td>–0.224*</td>
<td>–0.005</td>
<td>–0.156*</td>
<td>–0.081</td>
<td>–0.156*</td>
<td>0.123</td>
<td>0.034</td>
<td>0.158*</td>
<td>0.064</td>
<td>1.000</td>
</tr>
</tbody>
</table>

AAC, abdominal aortic calcification; ALP, alkaline phosphatase; BMD, bone mineral density; FN, femoral neck; hsCRP, highly selective C-reactive protein; LS, lumbar spine; PTH, parathyroid hormone; spKtV, single-pool Kt/V; TH, total hip.

*Data for hsCRP and AAC were log-transformed.

**p < 0.05.
Figure 1. Association of serum \( T_{50} \) with mineral and inflammatory markers in hemodialysis patients. Bivariate correlation analysis of serum \( T_{50} \) with (A) albumin, (B) phosphate, (C) calcium, (D) hsCRP, (E) AAC, and (F) BMD total hip. Serum \( T_{50} \) was positively correlated with serum albumin concentration \( (r = 0.289, p < 0.001) \) and inversely correlated with serum hsCRP \( (r = -0.224, p = 0.003) \) and phosphate \( (r = -0.156, p = 0.04) \) concentrations. AAC, abdominal aortic calcification; BMD, bone mineral density; hsCRP, high-sensitivity C-reactive protein.

Discussion

In this cross-sectional study of HD patients, \( T_{50} \) was associated with mineral and inflammatory parameters but not with AAC score or BMD.

CKD-MBD is a common complication of CKD and is associated with morbidity and mortality. The interconnection between vascular calcification and bone health has been reported as a significant inverse relationship between vascular calcification and bone fragility (low BMD) [16–18]. Impaired bone metabolism, particularly low bone turnover, may promote vascular calcification [1]. Several factors have been suggested as possible links between bone and soft tissue calcification; however, the key elements of the cross-talk mechanism are yet to be elucidated [1].

Reduced serum \( T_{50} \) is associated with a lack of inhibitors and abundant promoters of vascular calcification [19]. Therefore, it is assumed that the action of these factors and the effect of \( T_{50} \) on the overall tendency of serum calcification will be in the same direction.

The main determinants of \( T_{50} \) in this study were inflammatory (serum albumin and hsCRP), mineral (serum
phosphate), and the bone turnover marker (ALP). Only the values measured in the BMD total hip joint area showed a weak correlation in the unadjusted model, but there was no association with BMD measured in all regions in the multivariable-adjusted models. This finding is consistent with epidemiological data in advanced CKD-ND cohorts, where reduced T50 has been correlated with increased phosphate, decreased albumin, and CPP-associated fetuin-A concentration [8]. These results are also consistent with those of a dialysis cohort in which low BMD was related to mineral deposition in arterial walls and soft tissues [20,21].

Recently, an association between T50 and BMD was reported in 150 non-CKD participants from an elderly male cohort [22]. Subjects with a shorter T50 were likely to be older, and there was a nonlinear trend with a higher prevalence of diabetes, but T50 did not show any association with total hip or spine BMD. Moreover, there was no correlation with mineral parameters such as calcium and phosphate. The lack of association between T50 and BMD was consistent with our results, but the association between T50 and serum albumin from this and previous studies [8,9] could not be assessed.

A lower T50 was significantly associated with the severity and progression of coronary artery calcification in patients with CKD-ND; however, T50 was not associated with the incidence of coronary artery calcification [11]. In the present study, T50 was not associated with vascular calcification. The difference between these results is that although the range of our measurements using simple plain radiographs is limited, their correlation could be shown in their measurement methods using electron beam computed tomography. In addition, the fact that T50 is not related to the incidence of vascular calcification but correlates with its progression is thought to reflect a dynamic change in the progression of vascular calcification once it has occurred. This finding suggests that a significantly longer observation period is required to observe an association between T50 and vascular calcification.

The propensity for serum calcification reflects the degree of activity of numerous humoral and cellular factors that af-

**Table 3. Linear regression of the association between T50 (every 100 minutes increase) and BMD (g/cm²)**

<table>
<thead>
<tr>
<th>BMD</th>
<th>β (95% confidence interval)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spine L1–L4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>0.03 (–0.01 to 0.07)</td>
<td>0.10</td>
</tr>
<tr>
<td>Model 1</td>
<td>0.03 (–0.002 to 0.07)</td>
<td>0.07</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.02 (–0.02 to 0.06)</td>
<td>0.32</td>
</tr>
<tr>
<td>Model 3</td>
<td>0.02 (–0.02 to 0.06)</td>
<td>0.33</td>
</tr>
<tr>
<td>Femur neck</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>0.01 (–0.02 to 0.03)</td>
<td>0.71</td>
</tr>
<tr>
<td>Model 1</td>
<td>0.01 (–0.01 to 0.03)</td>
<td>0.42</td>
</tr>
<tr>
<td>Model 2</td>
<td>–0.001 (–0.02 to 0.02)</td>
<td>0.94</td>
</tr>
<tr>
<td>Model 3</td>
<td>–0.001 (–0.02 to 0.02)</td>
<td>0.96</td>
</tr>
<tr>
<td>Total hip</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>0.03 (0.001 to 0.06)</td>
<td>0.04</td>
</tr>
<tr>
<td>Model 1</td>
<td>0.03 (0.01 to 0.06)</td>
<td>0.01</td>
</tr>
<tr>
<td>Model 2</td>
<td>0.02 (–0.01 to 0.04)</td>
<td>0.19</td>
</tr>
<tr>
<td>Model 3</td>
<td>0.02 (–0.01 to 0.04)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Model 1: adjusted for age, sex, and smoking. Model 2: model 1 + adjustment for hemodialysis duration (mo), single-pool Kt/V, albumin, and alkaline phosphatase. Model 3: model 2 + adjustment for phosphate binders, vitamin D receptor activators, and cinacalcet.

BMD, bone mineral density.
fect the formation and growth of calcified crystals in blood vessels [7]. Calcification mechanisms require functional and direct measurements targeting calcium phosphate precipitation more comprehensively rather than focusing on the individual molecular components of the calcification process, as their developmental processes are multifactorial [8]. In this regard, it is considered that the contribution of $T_{30}$ could be large, and in the case of CKD patients, the results of a study comparing reduced $T_{30}$ and cardiovascular complications and death have been reported [8,9]. In patients with ESKD, an association between lower $T_{30}$, CVE, and mortality was reported in the EVOLVE (Effect of Cinacalcet on Cardiovascular Disease in Patients Undergoing Dialysis) study cohort [23]. However, in ESKD patients, dialysis itself and medications to maintain mineral parameters in the target range associated with CKD-MBD may affect $T_{30}$. Therefore, further studies are needed to evaluate its value as a predictor of clinical prognosis in patients with ESKD.

The T-score of BMD showed slightly different results depending on the measurement location, with the lowest values at the femur neck, the highest values at the lumbar spine, and a moderate level at the total hip. When studying the relationship between BMD and vascular calcification, there is currently no consensus as to which specific bone location should be the representative for BMD measurement [24]. This uncertainty is due to heterogeneity between the population and bone sites selected for BMD measurements in previous studies [16,25]. Depending on the severity of atherosclerosis, calcium deposition in the intima can affect the measurement of the spinal BMD. Our findings are consistent with those of previous studies that reported that peripheral BMD was lower than central BMD [26,27]. Lumbar BMD may be relatively underestimated in patients with ESKD with severe AAC. Therefore, peripheral BMD measurements may be more appropriate than central BMD measurements in these patients.

Osteoporosis causes both cortical and trabecular bone loss, whereas CKD-MBD results in primarily cortical bone loss [10,28]. In a recent study on the relationship between vascular calcification and BMD in CKD patients [29], cortical or trabecular bone loss was observed in CKD patients, but not all patients showed a simultaneous loss. In particular, the cortical bone loss did not show an association with vascular calcification, unlike trabecular bone loss [29]. Vascular calcifications are strongly associated with CKD-MBD [10,30]; however, the correlation between cortical bone and vascular calcification is not yet clear. In this respect, in this study, it is insufficient to explain the weak association between $T_{30}$ and BMD in the femur and the lack of association in the lumbar region. To evaluate the relationship between the pathophysiology of vascular calcification and bone density in HD patients with both MBD and osteoporosis components, consensus through follow-up studies on quantitative BMD measurement methods and sites according to pathophysiology is required.

This study has some limitations. First, a causal relationship could not be confirmed by conducting a cross-sectional study. However, we performed correlation analyses with various mineral parameters; in particular, we evaluated BMD in a relatively large number of HD patients, described its distribution, and analyzed its association with $T_{30}$. Second, we were unable to control the dialysis protocol and medications that affected $T_{30}$ measurements. However, considering that the characteristics of dialysis patients are always affected by medication as well as dialysis itself, we need to carefully consider the evaluation value of $T_{30}$ in future.

In summary, for the first time in Korea, we have provided a stable measurement method for $T_{30}$ and applied it to clinical research. $T_{30}$ was correlated with mineral and inflammatory parameters but not with AAC. BMD was correlated with $T_{30}$ in the case of total hip but was not correlated with BMD measured at other sites (femoral neck and lumbar spine). To evaluate the value of $T_{30}$ as a predictor of CKD-MBD diagnosis and treatment in ESKD patients, a study on its association with hard outcomes, including fracture, CVE, and mortality, should be prioritized. In addition, to confirm the association between $T_{30}$ and dynamic changes such as vascular calcification or BMD changes, a large-scale study that includes a larger number of patients and a longer observation period is needed.

**Conflicts of interest**

All authors have no conflicts of interest to declare.

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Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors’ contributions

Conceptualization: JYJ
Data curation, Formal analysis: HK, JYJ
Investigation: AJK, HR, JHC, HHL, WC
Methodology, Funding acquisition, Supervision: JYJ
Writing–original draft: HK, JYJ
Writing–review & editing: all authors
All authors read and approved the final manuscript.

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References

17. Naves M, Rodríguez-García M, Díaz-López JB, Gómez-Alonso C, Cannata-Andía JB. Progression of vascular calcifications is...
associated with greater bone loss and increased bone fractures. 


A 66-year-old man with end-stage renal disease due to cryoglobulinemia who was on hemodialysis received a kidney graft from a deceased donor. His prior medical history included human immunodeficiency virus (HIV) treated with dolutegravir and darunavir/cobicistat, past hepatitis C virus infection, and Child-Pugh-A-cirrhosis. Induction was based on basiliximab and maintenance on tacrolimus, mycophenolate, and prednisone. Transplantation was complicated by delayed graft function and irregular tacrolimus level due to antiretroviral interactions, with a final creatinine level of 3.5 mg/dL.

The patient was readmitted 2 months after transplantation for fever and vomiting with coffee-ground-appearing vomit. A diagnosis of urinary tract infection due to resistant Klebsiella pneumoniae was based on isolation of the microorganism both in the blood and urine; this infection was treated with meropenem. A blood analysis showed initial stability of renal function (creatinine, 3.3 mg/dL), low platelets (56,000/µL), leukocytes (5,900/µL), and elevated procalcitonin (11.66 ng/mL) and C-reactive-protein (CRP; 26.65 mg/dL). Urine sediment at admission was one to five red blood cells, leukocytes (20–30/µL), and positive nitrites and proteins (100–150 mg/dL) using test strips. Tacrolimus level was 12.8 ng/mL, and the patient was negative for cytomegalovirus. The results showed a BK virus charge and a nonreplicating HIV viral charge, with a CD4 level of 60 cells/µL. Upper endoscopy revealed esophageal candidiasis and an esophageal ulcer with Epstein-Barr virus replication. In consideration of the sepsis and the potential for opportunistic infections, the mycophenolic acid and tacrolimus were temporally suspended, while the steroid dose was increased.

After an initial improvement on day 10 with negative new blood cultures, the patient’s fever recurred; he became oliguric and required hemodialysis on day 16. While the procalcitonin decreased after antibiotic administration to 0.5 ng/mL on day 18, the CRP value remained elevated (7–13 mg/dL) and was classified as reactant without bacterial inflammation signs. Donor-specific human leukocyte antigen antibodies were not isolated by a Luminex test.
The differential diagnosis based on ultrasound, computed tomography (CT) scanning, and blood cultures was inconclusive, so an $^{18}$F-fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT scan was performed. It revealed intense and diffuse FDG uptake of the renal parenchyma (maximum standardized uptake value, 12.76), and there were no signs of infectious foci in the remainder of the study (Fig. 1A–C). A kidney biopsy was performed and revealed an acute T cell-mediated rejection of type IIB (Fig. 1D, E). By the time the biopsy was performed, urine sediment was negative for nitrites and bacteriuria, and there also was evidence of leukocyturia resolution, persistent microhematuria, and proteinuria of 150 to 300 mg/dL.

Immunosuppression was restarted with tacrolimus and
mycophenolate, and three boluses of 500 mg methylprednisolone were administered without clinical response. Antilymphocyte therapy was not considered due to the general state of the patient, and chronic dialysis was reinitiated.

In conclusion, even though further studies are needed, FDG-PET/CT could have a potential role in the diagnosis of kidney graft rejection as a noninvasive imaging method.

**Conflicts of interest**

The authors have no conflicts of interest to declare.

**Data sharing statement**

The data presented in this study are available on request from the corresponding author.

**Authors’ contributions**

Conceptualization: ABH, AGR, DC
Data curation: all authors
Methodology: AP, ABL
Writing–original draft: ABH, AGR, AP, ABL
Writing–review & editing: FC, FD, DC
All authors read and approved the final manuscript.

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Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is particularly life threatening in patients who are immunocompromised, including those with advanced chronic kidney disease (CKD) \[1,2\]. Despite the implementation of a third dose of a messenger RNA (mRNA) vaccine, the efficacy of SARS-CoV-2 vaccination on humoral and cellular immunities is reduced in the population with CKD, resulting in an increased incidence of severe infection and mortality, including in fully vaccinated patients \[3\].

In this context, several antiviral therapies or monoclonal antibodies are being investigated for treatment of COVID-19. These drugs prevent viral replication through various mechanisms, including neutralization, blocking SARS-CoV-2 entry, and inhibiting RNA polymerase or proteases activity \[4,5\]. However, patients with CKD are frequently excluded from clinical trials evaluating new drugs.

Although sotrovimab and casirivimab/imdevimab have been shown to confer satisfactory protection against the COVID-19 Delta variant, they have limited neutralizing activity against the Omicron variant \[4\]. Remdesivir, nirmatrelvir/ritonavir, bebtelovimab, and molnupiravir seem to be effective against the Omicron variant \[5\]. U.S. Food and Drug Administration product labels do not recommend remdesivir or nirmatrelvir/ritonavir in patients with an estimated glomerular filtration rate (eGFR) of <30 mL/min/1.73 m\(^2\) due to a lack of data concerning the risk of drug accumulation in this population. Indeed, nirmatrelvir and one of the excipients contained in remdesivir (betadex sulfobutyl ether sodium) are renally cleared and can accumulate in patients with abnormal kidney function. The appropriate dose for patients with severe renal impairment has not been determined. Bebtelovimab is only available in the United States. Thus, molnupiravir is the only antiviral drug that could potentially be used for CKD patients with the COVID-19 Omicron variant outside of the United States. Molnupiravir is an inhibitor of the RNA-dependent RNA polymerase of SARS-CoV-2. Although a phase III double-blind, placebo-controlled study of molnupiravir as an oral treatment for COVID-19 in nonhospitalized adults (MOVe-OUT) showed good efficacy, patients with eGFR of <30 mL/min or on dialysis were excluded \[6\]. To our knowledge, this is the first report on the efficacy and safety of molnupiravir in advanced CKD patients.

Three patients were on maintenance hemodialysis, one had received a transplant and had CKD G4 (eGFR, 18 mL/min/1.73 m\(^2\)), and one had CKD G5 (eGFR, 11 mL/min/1.73 m\(^2\)) (Table 1). Patients 1, 2, and 4 were under
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>56</td>
<td>71</td>
<td>46</td>
<td>60</td>
<td>57</td>
</tr>
<tr>
<td>Sex</td>
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<td>Male</td>
<td>Female</td>
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<tr>
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<td>FSGS</td>
<td>MCD</td>
<td>Tubulo-interstitial disease</td>
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<td>Dialysis modality (CKD stage)</td>
<td>In-center HD (CKD G5D)</td>
<td>No dialysis (CKD G4)</td>
<td>Home HD (CKD G5D)</td>
<td>No dialysis (CKD G5)</td>
<td>In-center HD (CKD G5D)</td>
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<td>CsA</td>
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<td>Reason for immunosuppressive therapy</td>
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<td>Renal transplant</td>
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<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<tr>
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<td>Type II</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>30</td>
<td>22</td>
<td>25</td>
<td>24</td>
<td>29</td>
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<td>Nodular regenerative hyperplasia</td>
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<td>No</td>
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<tr>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td>CLL</td>
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<td>Symptom</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cough</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>No</td>
<td>No</td>
<td>No</td>
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</tr>
<tr>
<td>Diarrhea</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Time between symptom onset and molnupiravir</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Vital signs at presentations</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Blood pressure (mmHg)</td>
<td>98/52</td>
<td>135/80</td>
<td>151/93</td>
<td>138/89</td>
<td>73/33</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>106</td>
<td>85</td>
<td>110</td>
<td>99</td>
<td>82</td>
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<td>Oxygen saturation (%)</td>
<td>93</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>98</td>
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<td>Need for supplemental oxygen</td>
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<td>No</td>
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<td></td>
<td></td>
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<tr>
<td>hsCRP (mg/L)</td>
<td>34</td>
<td>16</td>
<td>7</td>
<td>224</td>
<td>46</td>
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<tr>
<td>Platelets ($\times 10^3/\mu$L)</td>
<td>125</td>
<td>118</td>
<td>129</td>
<td>131</td>
<td>150</td>
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<td>Lymphocytes ($\mu$L$^{-1}$)</td>
<td>630</td>
<td>1370</td>
<td>410</td>
<td>770</td>
<td>770</td>
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<tr>
<td>eGFR (mL/min/1.73 m$^2$)</td>
<td>30</td>
<td>27</td>
<td>40</td>
<td>43</td>
<td>34</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
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<td>99</td>
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<td>SARS-CoV-2 variant</td>
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<td>SARS-CoV-2 viral load on nasopharyngeal swabs</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Day 0 (copies/mL)</td>
<td>20,335,411</td>
<td>18,222,021</td>
<td>4,700</td>
<td>1,761,144</td>
<td>43,275,421</td>
</tr>
<tr>
<td>Day 6 or 7 (copies/mL)</td>
<td>&lt;1,000</td>
<td>&lt;1,000</td>
<td>&lt;1,000</td>
<td>250,722</td>
<td>348,467</td>
</tr>
<tr>
<td>Day 13 (copies/mL)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1,685</td>
<td>2,071</td>
</tr>
<tr>
<td>Long-term prognosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>eGFR after treatment (mL/min/1.73 m$^2$)</td>
<td>NA</td>
<td>24</td>
<td>NA</td>
<td>19</td>
<td>NA</td>
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<tr>
<td>Remaining symptoms</td>
<td>Tiredness</td>
<td>No</td>
<td>No</td>
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</tbody>
</table>

CKD, chronic kidney disease; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CNI, calcineurin inhibitor; COVID-19, coronavirus disease 2019; Cs, corticosteroid; CsA, cyclosporin A; eGFR, estimated glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; HBP, high blood pressure; HD, hemodialysis; hsCRP, high sensitivity C-reactive protein; MCD, minimal change disease; MMF, mycophenolate mofetil; MPGN, membranoproliferative glomerulonephritis; NA, not available; NASH, nonalcoholic steatohepatitis; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Tac, tacrolimus.
immunosuppressive therapy (heart transplantation, renal transplantation, and treatment of minimal change disease, respectively). All five were fully vaccinated (four doses of the mRNA BNT162b2 vaccine [Pfizer-BioNTech]). They received molnupiravir at a dosage of 800 mg twice daily for 5 days (given after hemodialysis on dialysis day) for mild-to-moderate COVID-19. Quantitative reverse transcription polymerase chain reaction was performed on nasopharyngeal swabs at diagnosis, on day 6 or 7 to evaluate the time to clearance of the virus, and on day 13 for patients 4 and 5 (Fig. 1). Three patients showed <1,000 copies/mL at day 6 or 7. Patients 4 and 5 had results of 1,685 and 2,071 copies/mL on day 13, respectively, and rapid symptom resolution. No adverse effects were observed in any patient. Renal function remained stable in the two CKD patients who were not on dialysis (Table 1). One month after treatment, four patients were entirely asymptomatic and feeling well. Patient 1 still reported tiredness and loss of appetite. None of the five patients experienced delayed immune events or early recurrence of SARS-CoV-2 infection.

Molnupiravir is a produg that is metabolized to the ribonucleoside analogue N-hydroxycytidine (NHC). NHC is distributed into cells where it is incorporated into viral RNA by the viral RNA polymerase, which inhibits replication [7]. NHC is eliminated by cellular metabolism to uridine and/or cytidine through the same pathways involved in endogenous pyrimidine metabolism [8]. Renal clearance is not a meaningful route of elimination for NHC. For these reasons, no dose adjustments in patients with any degree of kidney impairment are recommended on the product label [7,8].

Molnupiravir is a safe drug with no contraindications (except during pregnancy and in patients aged <18 years because it may affect bone and cartilage growth). Side effects are limited; the most common (incidence ≥ 1%) include diarrhea, nausea, and dizziness [6]. No drug interactions have been identified (unlike for nirmatrelvir-ritonavir); although ritonavir is a potent CYP3A4 inhibitor and an inducer of other cytochrome p450 substances, oral administration allows treatment outside the hospital, while remdesivir or bebtelovimab requires an intravenous route.

In conclusion, this real-life observational study reported the safety of molnupiravir use in advanced CKD and its relative effectiveness on symptoms and virus clearance.

**Conflicts of interest**

All authors have no conflicts of interest to declare.

**Data sharing statement**

The data presented in this study are available on request from the corresponding author.

**Authors’ contributions**

Conceptualization: AD, LL
Data curation: ID
Investigation: ID, HG, PD
Formal analysis: ID, AD, EG, LL
Supervision and validation: LL, AD, EG, JDG
Ressources: AS, CB
All authors read and approved the final manuscript.

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References

1. Manuscript Submission

Manuscripts for *Kidney Research and Clinical Practice* (KRCP) should be submitted online at https://www.editorialmanager.com/krcp. All submissions to KRCP must conform to the International Committee of Medical Journal Editors (ICMJE) uniform requirements for manuscripts submitted to biomedical journals. Our requirements reflect those of the ICMJE, although we also have specific requirements for different types of article. For editorial questions, please contact us via e-mail (registry@ksn.or.kr), telephone (+82-2-3486-8736), or fax (+82-2-3486-8737).

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Articles should be prepared in the simplest form and submitted in the format of Microsoft Word (*.doc or *.docx). Manuscripts must be typed in English and double-spaced. All pages must be numbered consecutively starting from the title page. You may use automatic page numbering, but do NOT use other kinds of automatic formatting such as footnotes. Place text, references, tables and legends in one file with each table on a new page.

Please ensure that the following submission documents are also included, where applicable:

1. A cover letter. It must include your name, address, telephone and fax numbers, e-mail address, and state that all authors have contributed to the paper and have never submitted the manuscript, in whole or in part, to other journals.
2. A conflict of interest disclosure statement (see relevant section 4.2 below).
3. All studies involving human subjects, human data or any material derived from human must be approved by the relevant review or ethics committee. Articles must include a statement on ethics approval, the name of the relevant committee that approved the study and the committee’s approval number. Manuscripts may be rejected at any time if the authors of the research fail to provide the approval number validated by the relevant committee (see relevant section 4.1 below).
4. Articles covering the use of animals in experiments must be approved by the relevant authorities.
5. Articles where human subjects can be identified in descriptions, photographs or pedigrees must be accompanied by a signed statement of informed consent to publish (in print and online) the descriptions, photographs and pedigrees from each subject who can be identified.
6. The terms sex (when reporting biological factors) and gender (identity, psychosocial or cultural factors) should be correctly used. The sex and/or gender of study participants, the sex of animals or cells should be reported, and the methods used to determine sex and gender should be described. If the study was done involving an exclusive population, for example in only one sex, authors should justify why, except in obvious cases (e.g., ovarian cancer).
7. Clinical trials should be registered at a primary national clinical trial registration site such as www.clinicaltrials.gov, https://cris.nih.go.kr/cris/index.jsp, or other sites accredited by the World Health Organization or the International Committee of Medical Journal Editors.
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9. Articles should be written in English (using American English spelling) and meet the following basic criteria: the material is original; the information is important; the writing is clear, concise and grammatically correct; the study methods are appropriate; the data are valid; and the conclusions are reasonable and supported by the data. The articles should be readable to native English users, and we recommend using professional language editing service (e.g., American Journal Experts) prior to submission to avoid delays with the review processes.
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2. Types of Articles

2.1. Original Articles

These are expected to present major advances and important
new research results. Section headings should include Abstract, Introduction, Methods, Results, Discussion, Conflicts of interest, Acknowledgments (if applicable), and References. The text should be limited to 4,000 words (excluding tables, figures and references) and 40 references.

2.2. Review Articles
These describe new developments of significance in the field of nephrology and highlight unresolved questions and future directions. Most reviews are solicited by the editors, but unsolicited submissions may also be considered for publication. Review articles should include Abstract, Introduction, brief main headings, and References. The text should be limited to 5,000 words (excluding tables, figures and references) and 100 references.

2.3. Special Articles
Articles in this section should provide insightful analysis and commentary about any important topic in medicine, research, ethics, or health policy. They may also address consensus statements, guidelines, statements from task forces, or recommendations. Most reviews are solicited by the editors, but unsolicited submissions may also be considered for publication. The text should be limited to 5,000 words (excluding tables, figures and references) and 50 references.

2.4. Correspondence
Correspondence generally takes one of the following forms: (1) Reader’s comment on an article previously published in KRCP and/or a reply from the authors; (2) An article that may not fit to the format of original or review article but suggest creative perspectives for medical issues; (3) A brief report of any kind that presents important research findings adequate for the journal’s scope and of particular interest to the readers. The submitted manuscript includes title page, main text, conflict of interest, acknowledgments (if applicable) and references. No abstract is included, and the text should be limited to 800 words (excluding tables, figures and references) and 8 references. A maximum of 2 figures or tables may be included.

2.5. Editorials
These are manuscripts that are related to materials within the current issue; they raise challenging questions or explore controversies. The editor solicits such opinion pieces. The order of the submitted manuscript includes title page, integrated discussion, conflict of interest, acknowledgments (if applicable) and references. The text should be limited to 1,500 words and 10 references. A maximum of 2 figures or tables may be included.

2.6. Images in Practice
These present classic or unique images of common medical conditions in clinical nephrology. Images are an important part of much of what we do and learn in clinical practice. The text should be limited to 400 words. There should be no more than two figures. No tables or references are included.

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3.1. Title Page
The title page should include article title, each author’s first and last names, positions (associate professor, fellow, student, etc.), and ORCID identifiers, and the institutions with which they are affiliated, short running title not exceeding 50 characters, separate word count for abstract and text, and details of the corresponding author (name, address, phone, and e-mail information). Funding sources should be included, and the individual contribution of each co-author must also be detailed (see relevant section 4.3 below).

3.2. Abstract and Keywords
Abstract should not exceed 250 words in original, review or special articles. It must be written for easy reading with no abbreviations. The abstract of the original article should be divided into four subsections: Background, Methods, Results, and Conclusion. Four to six keywords should be listed alphabetically below the abstract. For selecting keywords, refer to the Index Medicus Medical Subject Headings (available from: http://www.ncbi.nlm.nih.gov/mesh).

3.3. Main Text
The text for original articles, for example, should include the following sections: Introduction, Methods, Results, and Discussion. The Introduction should be as concise as possible, without subheadings. The Methods section should be sufficiently detailed. Subheadings may be used to organize the Results and Discussion. Each section should begin on a new page.

3.4. Acknowledgments
General acknowledgments for consultations, statistical analysis and so on should be listed after main body of text, before the References section, including the names of the individuals involved. All financial and material support for the research
and the work should be stated here clearly and explicitly.

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References should be cited with Arabic numerals in square brackets. References are numbered consecutively in order of appearance in text. References are limited to those cited in text and listed in numerical order. List all authors if there are less than or equal to six authors. List the first three authors followed by “et al.” if there are more than six authors. If an article has been published online but has not yet been given an issue or pages, the digital object identifier (DOI) should be supplied. Journal titles should be abbreviated in the style used in Index Medicus. Other types of references not described below should follow The NLM Style Guide for Authors, Editors, and Publishers (https://www.ncbi.nlm.nih.gov/books/NBK7256/). The authors may format the citations and references using the KRCP EndNote style file, but we generally recommend the authors to type the citation numbers and references manually.

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Tables are numbered consecutively using Arabic numerals in the order of their citation in text. Table titles should be short and descriptive (e.g. Table 1. Demographic characteristics of patients). If numerical measurements are given, the unit of measurement should be included in the column heading. The statistical significance of observed differences in the data should be indicated by the appropriate statistical analysis. All nonstandard abbreviations should be defined in footnotes. Lower case letters in superscripts (\( a, b, c \)) should be used for special remarks.

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Figure legends should be submitted for all figures. They should be brief and specific, and placed on a separate sheet after the References section. Figures are numbered consecutively using Arabic numerals in the order of their citation in the text. Figures should be uploaded as separate files, not embedded in the manuscript file. Figures that are line drawing or photographs must be submitted separately in high-resolution EPS or TIFF format (or alternatively in high-resolution JPEG format). Only high-resolution figure files (preferably 300 dpi for color figures and 1,200 dpi for line art and graphs) should be submitted. The files are to be named according to the figure number and format (e.g., Fig1.tif). Figures that are reproduced from other published sources require written permission from the authors and copyright holders.

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参考文献

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2. Jardiance Duo (empagliflozin/metformin-HCl) 10mg/500mg, 10mg/1000mg
3. Jardiance Duo (empagliflozin/metformin-HCl) 25mg/500mg, 25mg/1000mg
4. Jardiance Duo (empagliflozin/metformin-HCl) 50mg/500mg, 50mg/1000mg
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경구 혈당감히
세라 초기 용량
2배에 해당하는 용량으로 1개월에 1회씩 투여받을 수 있다. 2. 조혈촉진제를 투여받고 있는 환자: 현재 다른 조혈촉진제를 투여받고 있는 환자에 이 약을 1개월 1회 대체 투여할 수 있다. 이 약의 초기 용량은 표 1과
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약 1~2정(포)을 다음과 같이 혈청 인 수치에 따라 매 식사와 함께 복용한다. 혈청 인 5.5 - 7.5 ㎎/dL의 경우 1회 1정(포), 1일 3회, 7.5 ㎎/dL이상의 경우 1회 2정(포), 1일 3회. 2) ... 환자에서 이 약을 대체 투여: 동일 용량을 투여한다. 투석을 받는 만성신장질환 환자에서 연구된 세브라머 탄산염의 최대 1일 용량은 14g이었다. 3) 세브라머 탄산염의 정제에서 산제로 또는 산제에서 정제로 대체투여:


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1. Anemia
2. Chemotherapy-induced anemia in solid cancer patients

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- Initiating patients
  - Initial dose
    The usual dose of NESP in adult patients is 40 μg to be administered as a single intravenous injection once weekly. If there is a need to escalate the dose, the frequency of administration can be increased up to once every two weeks. The initial dose may be increased by 10 μg per week until the desired response is achieved.

- Maintenance dose
  When a desired response is achieved, the usual dose of NESP in adult patients is 80 μg to be administered as a single intravenous injection once weekly. If there is a need to escalate the dose, the frequency of administration can be increased up to once every two weeks. The initial dose may be increased by 10 μg per week until the desired response is achieved.

Precautions related to Dosage and Administration

1. Initial dose at the switching from erythropoietin preparations: See Precautions related to Dosage and Administration
2. Maintenance dose
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Boryung Renal Business Unit provides **TOTAL RENAL CARE**

<table>
<thead>
<tr>
<th>Boryung Renal Business Unit</th>
<th>Boryeong Building, 136 Changgyeonggung-ro, Jongno-gu, Seoul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Customer Service Center</td>
<td>Tel 080.708.8088 Fax 02.741.5291 <a href="http://www.boryung.co.kr">www.boryung.co.kr</a></td>
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