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OPINION REVIEW

Ultrasound, contrast-enhanced ultrasound and pyelonephritis: A narrative review

Andrea Boccatonda, Roberta Stupia, Carla Serra

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Abstract

Acute pyelonephritis (APN) is a bacterial infection resulting in kidney inflammation, typically arising as a complication of an ascending urinary tract infection that ascends from the bladder to the kidneys. Clinical diagnosis is generally based on clinical and laboratory findings. Recent guidelines recommend not performing diagnostic imaging unless a complicated APN is suspected or the infection affects high-risk patients such as the elderly, immunocompromised individuals, or diabetics. Contrast-enhanced ultrasound (CEUS) is a valuable tool in both the diagnosis and follow-up of APN. It aids in distinguishing small simple nephritic involvement from abscess complications and monitoring their evolution over time during antibiotic therapy. Given its lack of ionizing radiation and nephrotoxicity, CEUS is a valid diagnostic modality for approaching and monitoring pyelonephritis, improving early identification and characterization of inflammatory lesions. This review aims to summarize the main evidence on the use of ultrasound and CEUS in the diagnosis of APN and its follow-up.

Key Words: Pyelonephritis; Contrast-enhanced ultrasound; Kidney; Abscess; Urinary infection

Core Tip: Acute pyelonephritis (APN) is a kidney inflammation typically due to an ascending urinary tract bacterial infection. The diagnosis is suggested by clinical and laboratory findings. Diagnostic imaging should be performed in case of a complicated APN. Contrast-enhanced ultrasound (CEUS) is a valid diagnostic tool for evaluating APN and performing a follow-up. This review aims to summarize the main evidence on the use of ultrasound and CEUS in the diagnosis of APN and its follow-up.

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INTRODUCTION

Acute pyelonephritis (APN) is a bacterial infection resulting in kidney inflammation, typically arising as a complication of an ascending urinary tract infection (UTI) that ascends from the bladder to kidneys[1]. In the United States, the estimated annual incidence of APN ranges from 459000 cases to 1138000 cases, with 10.5 million to 25.9 million cases occurring globally each year[2]. The most commonly affected group is young, sexually active women, with almost all cases related to *Escherichia coli* infection[3]. Conversely, pyelonephritis in men > 18 years, elderly women, subjects affected by urological issues, and hospitalized individuals often results from less virulent *E. coli* strains, other Gram-negative bacilli, Gram-positive bacilli, and *Candida*[3]. The most common symptoms encompass fever, pain on the flank, nausea and vomiting, dysuria, and pollakiuria[3]. If there is no clinical improvement after 72 hours of intravenous antibiotic therapy or if a urinary tract obstruction is present complications should be considered. Clinical diagnosis is generally based on clinical and laboratory findings; a urinalysis and culture should always be performed in patients suspected of APN before administering antibiotics. Nevertheless, urine culture may be negative in up to 30% of pyelonephritis cases, possibly due to the administration of outpatient antibiotics[4]. Blood exams such as a complete blood cell count are sent to look for leukocytosis and laboratory signs of sepsis. Creatinine and blood urea nitrogen should be required to assess kidney function. There are no serum biomarkers available specific for pyelonephritis, although urinary neutrophil gelatinase-associated lipocalin may be a useful and sensitive indicator of APN in children and possibly in adults[5,6]. If a complicated APN is suspected or if the patient falls into high-risk categories, such as immunocompromised individuals, elderly or diabetics, diagnostic imaging should be performed[7].

ROLE OF ULTRASOUND

The European Association of Urology guidelines on urological infections published in 2024 indicate that ultrasound should be performed to exclude obstruction of the urinary tract or the presence of renal stones[8]; additional investigations such as contrast-enhanced computed tomography (CT) or urography should be considered in patients who remain febrile and/or do not improve after 72 hours of therapy or in those with worsening clinical status[8]. Moreover, ultrasound or resonance imaging is suggested for the diagnosis of complicating factors during pyelonephritis in pregnant women[8]. As reported by the American College of Radiology, CT abdomen and pelvis without and with contrast is usually appropriate for the initial imaging of complicated patients with suspected APN[9]. The use of routine United States for APN is questioned as reported in a recent systematic review and meta-analysis by Yu *et al*[10]. Therefore, there are few discordant data, and there is no mention of ultrasound as the first evaluation/screening method for suspected pyelonephritis, much less for contrast-enhanced ultrasound (CEUS) in the guidelines.

In our previous experience, CEUS was used as the first and only method of evaluating suspected pyelonephritis in 35.7% of cases[11]. In our opinion, these data are even more relevant in clinical reality, as in our work, many examinations were performed as controls on patients who came from other hospitals where it was not possible to perform a CEUS. In some cases, the minority, both CEUS and contrast-enhanced CT were requested due to clinical-radiological inconsistencies (14.2%)[11].

Diagnostic imaging can evaluate the location and extent of lesions and indicate possible underlying causes[7]. Despite the B-mode ultrasound can detect alterations in pyelonephritis with complications, it is less accurate than CT, which is still the preferred diagnostic tool[12]. Due to concerns about radiation exposure and potential nephrotoxicity, CEUS has emerged as an alternative imaging modality for accurately detecting renal abnormalities in APN[12]. A 2007 study demonstrated that CEUS has a diagnostic performance similar to that of CT, particularly for focal involvement[13]. The 2017 update from the European Federation of Societies for Ultrasound in Medicine and Biology recommends CEUS for assessing renal abscesses in complicated APN[14]. However, renal abscesses represent only one possible inflammatory alteration in the renal parenchyma due to APN.

ULTRASOUND EXAMINATION TECHNIQUE

On B-mode ultrasound, the kidneys are examined using axial and longitudinal scans[15]. B-mode exploration helps to exclude hydronephrosis and to evaluate echo-structural pathological changes, such as perirenal fluid collections[15]. This assessment is complemented by a color-Doppler evaluation[15].

Abnormalities detected by color and power Doppler sonography in a cohort of infants with APN poorly correlated with Tc-99 m dimercaptosuccinic acid (DMSA) renal scintigraphy findings[16]. However, the sensitivity of color and power Doppler sonography is highly age-related and can be a non-invasive helpful tool for early diagnosis of APN in infants older than 6 months[16].

Moreover, microvascular Doppler ultrasonography showed improved detectability of hypoperfused areas in pediatric APN, providing higher diagnostic confidence[17].

Kidneys affected by APN showed a mean resistive index (RI) of 0.744 ± 0.06 in infants, 0.745 ± 0.03 in preschool children, and 0.733 ± 0.09 in school-age patients with upper UTI[18]. The mean RI values were significantly higher in patients with upper UTI ($P < 0.001$)[18]. There was a highly significant correlation between RI values and the severity of the renal lesion as ranked by DMSA scintigraphy ($P < 0.001$)[18]. When the cut-off RI value was 0.715, there was an 80% sensitivity and an 89% specificity for diagnosing upper UTI. Refluxing kidneys and scarred kidneys also had higher RI values[18].

In another study, kidneys with permanent damage had a mean RI of 0.71 ± 0.06, while the RI value for non-scarred kidneys was 0.66 ± 0.06 ($P = 0.02$)[19]. The best cutoff point of the RI value was 0.715, with a sensitivity of 70%, a specificity of 87.7%, and positive and negative predictive values of 32% and 97%, respectively[19].

After completing the B-mode exam, the CEUS study can be conducted. A sulfur hexafluoride-based contrast medium, SonoVue® (Bracco, Italy), is rapidly injected intravenously at a volume of either 2.4 mL or 4.8 mL, followed by a 10 mL saline flush. Following the ultrasound contrast agent bolus injection, the kidneys enhance quickly and intensely. The contrast enhancement is visible in the arterial vessels 10-15 seconds following injection, and then by the renal cortex a few seconds later, while the pyramids remain not perfused. The kidney pyramids gradually present a wash-in, thus displaying the same perfusion with the cortex within 30-40 seconds following SonoVue® infusion. Initially, the kidneys appear hyperechoic compared to the liver or spleen. Subsequently, the kidneys display lower perfusion than the near parenchyma, particularly the spleen (Figure 1)[20]. Since SonoVue® is not metabolized and excreted in the urinary system, the kidney collecting system never presents contrast enhancement.

B-mode ultrasound and color-power-Doppler in APN

Cortical round hyperechoic lesion is the most frequent ultrasound finding in complicated APNs, typically accompanied by cortical bulging. This feature is characteristic of APNs, and it can also be detected in APNs with small-size abscesses. Larger abscesses are characterized by inhomogeneous or hypoechoic areas and cortical bulging. Color-power-Doppler evaluation reveals these pathological areas as low-perfused lesions[21]. B-mode ultrasound is less sensitive and specific compared to other diagnostic imaging modalities, failing to detect APN lesions in up to 50% of patients[22,23]. The application of power-Doppler ultrasound displays a sensitivity of 89% and specificity of 53% in identifying kidney inflammatory changes[24]. Additionally, ultrasound with tissue harmonic imaging is characterized by a sensitivity of 97% and specificity of 80% in the evaluation of APNs[25].

CEUS in APN

Depending on the kidney involvement, APNs may be categorized as focal, multifocal, or diffuse. In the diffuse form of APN, CEUS diagnostic accuracy appears to be lower if compared to CT because it does not allow simultaneous comparison with the contralateral kidney (Table 1). However, CEUS has demonstrated very high diagnostic accuracy for evaluating focal and multifocal forms of APN, with a positive predictive value of 100% and a negative predictive value of 89%[12,13,21].

Focal pyelonephritis presents as wedge-shaped and/or round hypoechoic cortical lesions or affecting both the cortex and medulla (Figure 2). In terms of enhancement patterns, these lesions are most clearly seen during the late parenchymal phase, with variable findings during other phases.

Typically, the lesions are low perfused in the early phase, then become isoechoic to kidney parenchyma, and finally return to being hypoechoic in the late phase. Moreover, the abscess appears as a non-perfused area in all phases with a round shape; a peripheral rim enhancement may be observed in the early phase.

Specific categories of patients

In the context of transplanted kidneys, CEUS has shown significant value for the early diagnosis of APN. It helps to limit the utilization of iodinated or paramagnetic contrast media, thereby preventing the risk of kidney damage and toxicity, particularly in subjects with chronic kidney disease[26,27]. In a work by Granata *et al*[23], 56 subjects with suspected APN in transplanted kidneys were evaluated by CEUS in comparison with magnetic resonance imaging (MRI) with gadolinium. The study demonstrated good diagnostic accuracy for CEUS, with a sensitivity of 95% and specificity of 100%[23]. CEUS can identify early complicated lesions such as acute focal bacterial nephritis (AFBN), enabling the selection of an appropriate therapeutic regimen. In a study by Hosokawa *et al*[28], involving a small cohort of pediatric patients with UTIs, ultrasound proved feasible in differentiating AFBN from APN, using contrast-enhanced CT as the reference standard. Notably, the study found a significant difference in the presence of ultrasound-detected focal loss of corticomedullary differentiation among subjects with AFBN and those with APN ($P = 0.01$)[28].

Table 1 Studies published assessing B-mode and contrast-enhanced ultrasound in patients with pyelonephritis (order by publication year)

Ref.	Year	Country	PMID	Study design	Aim of the study	Sample size	Mean age in years	Sensitivity	Specificity
Kim *et al*[25]	2001	Korea	11149528	Prospective study	CEUS in APN compared with CT	30		NA	NA
Stunell *et al*[7]	2006	Ireland	16937102	Comparative study	CEUS in APN compared with CT				
Mitterberger *et al* [13]	2007	Austria	17941932	Prospective study	CEUS in APN compared with CT	100	30.2	98	100
Granata *et al*[23]	2011	Italy	20659906	Prospective study	CEUS in APN compared with MRI	56	50.1	95	100
Fontanilla *et al* [21]	2012	Spain	21792579	Observational study	CEUS in evaluation and follow-up of APN	48		NA	NA
Hosokawa *et al* [28]	2020	Japan	32162084	Observational study	US compared with CT in AFNB or APN	11			
Boccatonda *et al* [11]	2023	Italy	37958043	Retrospective study	CEUS as follow-up of APN	28	49.2	NA	NA

AFBN: Acute focal bacterial nephritis; APN: Acute pyelonephritis; CEUS: Contrast enhanced ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging.

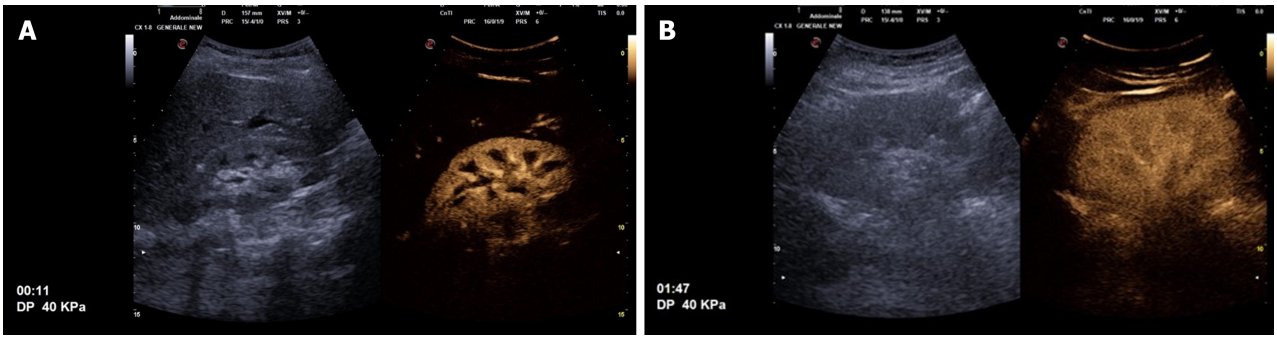

Figure 1 Following the ultrasound contrast agent injection. A: Enhancement in the central arteries becomes visible 10-15 seconds after contrast injection, followed by the renal cortex a few seconds later, while the pyramids remain echo-poor; B: The renal pyramids gradually fill in, becoming almost isoechoic with the cortex within 30 seconds to 40 seconds after injection. During early-phase scanning, the kidneys appear hyperechoic compared to the liver or spleen. Later, the kidneys turn rapidly hypoechoic relative to the adjacent parenchyma, particularly the spleen.

In the emergency setting, the early detection of those findings by CEUS can help to reduce exposure to radiation and iodinated contrast medium related to CT, which is particularly beneficial for pediatric patients.

Furthermore, CEUS can be used to evaluate the right placement of a nephrostomy tube by administering the contrast medium into the drainage catheter. This technique allows for the verification of unobstructed drainage, ensuring the proper function of the nephrostomy tube[29].

APPLICATION OF ULTRASOUND AND CEUS IN THE CLINICAL SCENARIO OF PYELONEPHRITIS

Ultrasound is typically the initial diagnostic imaging used in the assessment of patients suspected of having upper UTIs. However, the signs of renal infection are often not assessable on B-mode ultrasound, so diagnosis primarily relies on clinical and laboratory data along with indirect ultrasound findings[1]. In cases of complicated APN, ultrasound imaging is employed to ascertain the involvement of the inflammatory process over the kidney or to detect ureteral obstruction [12].

Historically, CT is considered the gold-standard imaging tool for diagnosing APN complications[12]. Otherwise, CT displays notable drawbacks, including exposure to radiation, especially concerning young patients, as well as the potential nephrotoxicity of contrast media.

While CT remains valuable in diagnosing complicated APN, the drawbacks associated with radiation exposure and contrast media toxicity have led to a growing interest in alternative imaging modalities. CEUS has emerged as a promising tool, offering excellent diagnostic accuracy comparable to CT but without the associated radiation exposure or

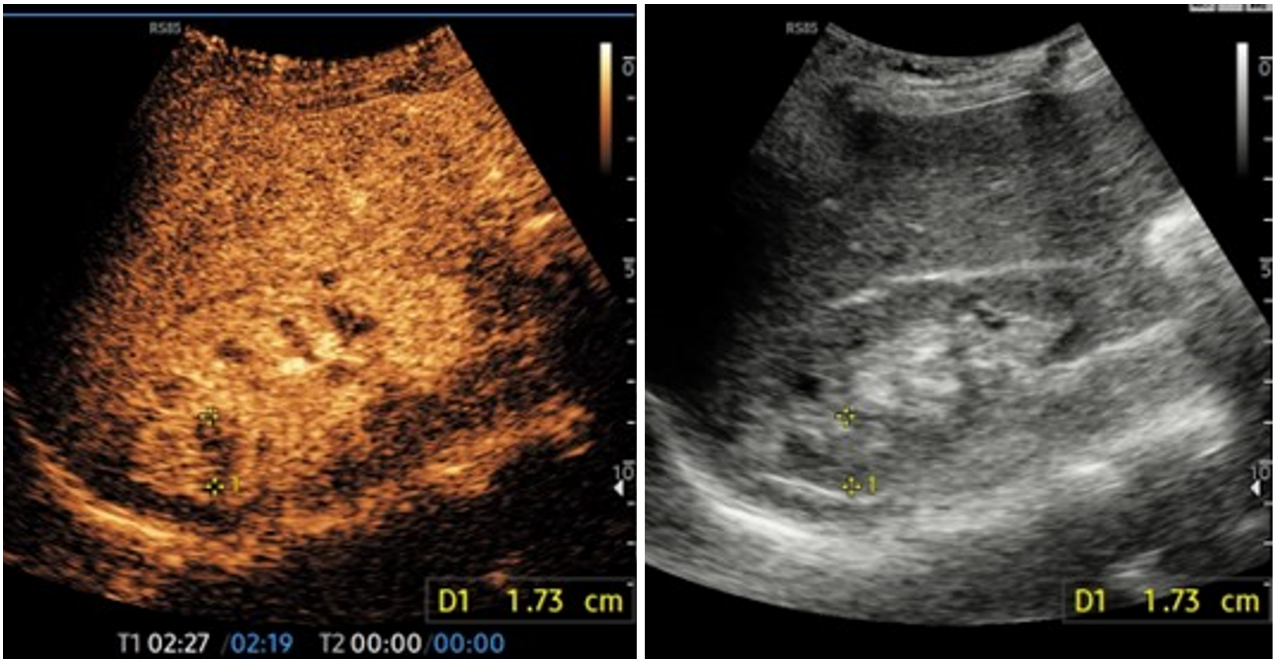

Figure 2 B-mode and contrast enhanced-ultrasound images showing a focus of pyelonephritis. In the B-mode image, a non-homogeneous ovary image is highlighted at the cortical level. Upon completion of contrast enhanced-ultrasound, this area appears hypovascular compared to the surrounding parenchyma, especially 40 seconds after the infusion of the contrast medium.

risk of contrast-induced nephrotoxicity. CEUS provides detailed visualization of renal parenchymal perfusion and can effectively identify early complications of APN, aiding in timely and accurate diagnosis without the drawbacks of CT.

In this scenario, CEUS assumes a pivotal role due to its avoidance of ionizing radiation and non-nephrotoxic contrast agents. Recent works showed a comparable diagnostic accuracy of CEUS to CT in evaluating kidney parenchymal changes related to pyelonephritis[13,30]. CEUS can depict cortical areas with low perfusion related to infection, with studies indicating high accuracy and a 100% PPV for APN diagnosis, with CT scan as the reference standard[7].

Fontanilla *et al*[21] provided detailed descriptions of CEUS findings in APN, offering insights into typical enhancement features of various parenchymal lesions. This enables differentiation between abscesses and focal pyelonephritis and facilitates the detection of even small abscesses within pyelonephritic areas[21]. This highlights the valuable diagnostic capabilities of CEUS in the assessment of APN, allowing for precise characterization of lesions without the drawbacks associated with CT imaging.

In the emergency setting, CEUS exhibits high sensitivity and specificity in diagnosing renal infarction[31]. CEUS is increasingly utilized for distinguishing kidney infarction and necrotic areas, particularly when vascular disease is suspected to induce acute kidney failure[31]. Additionally, time-intensity curve software allows quantification of renal perfusion, offering a reliable tool for post-therapeutic monitoring to assess residual inflammatory infiltrate[32].

For patients with contraindications to contrast media administration or transplant recipients, diffusion-weighted (DW) MRI emerges as an optimal diagnostic tool for identifying APNs. Research by Faletti *et al*[33] investigated the role of DW MRI in managing APN in transplanted kidneys. Their study revealed significant differences in apparent diffusion coefficient parameters between normal cortical areas and APNs, and to differentiate APN from abscesses[33]. DW MRI thus proves to be a valuable tool in the diagnosis and management of APN, particularly in populations with specific contraindications or considerations regarding contrast media usage[33].

Focal pyelonephritis typically presents as a hyperechoic focal lesion with cortical bulging on grey-scale ultrasound, related to the high number of inflammatory cells in the interstitium and tubules[30]. Moreover, relevant capillary damage in association with leukocyte infiltration and fibrin plugs has been observed[12]. Those tissue changes are responsible for the hypoechoic feature and reduced enhancement of APN areas on CEUS, compared to healthy kidney. Focal APNs are better detectable in the late phase of CEUS, as they appear more hypoechoic during this phase.

Abscesses manifest as inhomogeneous and/or hypoechoic areas with cortical bulging. Ultrasound and CEUS enable the assessment of measure, structure and extent of abscesses. Since an abscess is a necrotic cavity containing pus and debris, no enhancement is observed inside throughout all phases. However, enhancement of septa may be visible if the abscess is partially liquefied. Peripheral rim enhancement in the cortical phase may also be observed.

The morphological features observed on CEUS in complicated APN are highly specific, leading to few potential differential diagnoses. When distinguishing between focal APN, abscesses, and infarcts, it's crucial to consider the enhancement pattern. Unlike APN, infarcts are characterized by non-enhancing areas. Additionally, the morphology of the lesion aids in the differentiation between infarcts and abscesses; infarctions typically exhibit a wedge-shaped appearance, whereas abscesses present as round, geographical, or coalescent lesions. Infarctions usually do not cause cortical bulging, whereas cortical bulging is frequently observed in large or medium-sized pyelonephritis, with or without abscesses[7]. Furthermore, pyelonephritis can lead to scars that are indistinguishable from those caused by infarctions.

In cases of peri- and para-renal involvement, patients typically undergo CT or MRI. MRI is suggested for young subjects with peri-renal disease extension. It's important to note that SonoVue™ is not related to kidney damage, and it can be administered to subjects with reduced renal function and in renal transplant recipients safely. Caution should be exercised in pregnant and lactating patients, as there is currently no available clinical data about the use of CEUS in those populations.

FOLLOW-UP OF APN

CEUS plays a crucial role in the follow-up of patients with complicated APN. Utilizing CEUS for follow-up examinations reduces the patient's exposure to a significant dose of radiation[11,34].

The European Association of Urology guidelines on urological infections published in 2024 do not give any recommendations on possible post-therapy imaging follow-up. This contrasts with the clinical reality where patients are often re-evaluated after a few weeks both *via* B-mode and CEUS methods. According to Rinaldo *et al*[34], follow-up examinations typically occur at 1-2 weeks, and 1-month intervals, depending on the type of initial lesion and the response to antibiotic therapy.

In our previous work, all patients who underwent a CEUS follow-up for APN experienced resolution of the lesion[11]. With the limitations of the retrospective nature of the study, the times of follow-up and therefore ultrasound demonstration of healing of the renal lesion were very heterogeneous[11]. In general, it was possible to assert not to set up an ultrasound check too early, but to set it up at least 25 days after the first diagnosis of pyelonephritis; this is to wait a suitable time for the lesion to heal and therefore reduce the number of tests to be performed[11].

Furthermore, it was interesting to note that the ultrasound findings did not prompt any therapeutic modifications or lead to the prescription of additional laboratory tests in any instance[11].

This certainly raises doubts about the real clinical usefulness of CEUS control, if we exclude the desire to have imaging confirmation of the complete healing of the affected portion of the kidney.

Despite its advantages, CEUS has limitations, primarily related to patient factors such as body habitus and operator experience. Issues like obesity and bowel gas can affect the image quality of parenchymal perfusion, and those factors are crucial in determining the best diagnostic strategy for those subjects (CEUS *vs* CT or MRI).

CONCLUSION

In conclusion, CEUS proves to be a valuable technique in the diagnosis and follow-up of APNs. It aids in distinguishing focal pyelonephritic lesions from abscesses and monitoring their changes after antibiotic therapy. However, it is essential to acknowledge that CT and MRI continue to play a crucial role in evaluating peri-renal complications. The major limitation of the CEUS method compared to CT is certainly the poor panoramic view and the difficulty in evaluating both kidneys, especially if the symptoms are not localized, with a single bolus of contrast. Future studies will have to evaluate in which categories of patients reporting symptoms of UTI it is appropriate to perform an ultrasound and a CEUS. Furthermore, it is essential to understand the timing of the first imaging examination in relation to the onset of symptoms and subsequently set the correct follow-up time.

FOOTNOTES

Author contributions: Boccatonda A was responsible for the conceptualization; Stupia R was responsible for the writing and editing; Serra C was responsible for expert review; All authors have read and approved the final manuscript. Boccatonda A and Stupia R collaborated equally in the study conception, data collection and manuscript writing efforts that were crucial to this study's successful completion, meriting the co-first authorship designation.

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REVIEW

Insights into renal and urological complications of inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a chronic condition characterized by immune-mediated inflammation in the gastrointestinal tract, which follows a relapsing and remitting course. Apart from affecting the gastrointestinal tract, IBD also has extra-intestinal manifestations (EIMs). While the etiology of extraintestinal manifestation remains unclear, it is theorized to be based on immunological responses influenced by genetic factors. Renal involvement is one of the EIMs observed in ulcerative colitis and Crohn's disease. The renal manifes-tations in IBD patients encompass a range of conditions including nephrolithiasis, amyloidosis, tubulointerstitial nephritis, glomerulonephritis (GN), obstructive pathologies, and chronic kidney disease (CKD). The incidence of CKD in IBD patients varies from 5%-15%. The decline in renal function can stem from various factors such as direct inflammatory damage to the kidneys leading to glomerular or tubular injury, or from complications like recurrent stones, amyloidosis, or GN.

Additionally, nephrotoxic medications used in treating IBD, such as TNF-α inhibitors, calcineurin inhibitors, and aminosalicylates, can exacerbate the decline in renal function. Currently, there is a lack of consensus regarding these patients' screening and renal function monitoring. This review aims to assess the existing literature on the different renal complications among individuals with IBD, shedding light on their pathophysiology and management.

Key Words: Inflammatory bowel disease; Glomerulonephritis; Amyloidosis; Extra-intestinal manifestations; Nephrotoxicity; chronic kidney disease

Core Tip: Renal manifestations have been described in 4%-23% of the patients with Inflammatory bowel disease, leading to significant morbidity and mortality. Glomerulonephritis, nephrolithiasis, amyloidosis, and tubulointerstitial nephritis are common with approximately 5%-15% of the patients developing chronic kidney disease. Serum markers such as creatinine levels, especially cystatin-c levels, should be used to monitor renal function, especially in patients receiving nephrotoxic medications such as aminosalicylates and TNF-α inhibitors.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a debilitating chronic inflammatory disorder of the gastrointestinal tract, with a global disease burden of around 4.9 million cases[1]. Besides affecting the intestine, this disease can also present with extra-intestinal manifestations (EIMs), the prevalence of which varies from 6% to 46%[2]. The most common EIMs reported in the literature are joint (peripheral and axial arthropathies), skin (erythema nodosum, aphthous ulcers, pyoderma gangrenosum), biliary tract (primary sclerosing cholangitis, peri cholangitis), and eye (uveitis, scleritis) involvement[3,4]. In addition to these, renal manifestations have also been described in around 4%-23% of the patients with IBD[5,6].

Nephrolithiasis, amyloidosis, tubulointerstitial nephritis, glomerulonephritis (GN), amyloidosis, fistulas, and urothelial cancers are common renal manifestations (Figure 1). Similar to other EIMs, the pathogenesis of renal EIMs is unclear. Immune response dysregulation, susceptible loci, and shared biological pathways are all expected to play a role[7]. Furthermore, treatment for IBD requires various immunomodulators that can be nephrotoxic and can lead to a reduction in kidney function.

As a result, IBD is considered to be a risk factor for the progression of chronic kidney disease (CKD). A prospective study by Liu *et al*[8] reported patients with IBD to have 57% and 96% higher odds of developing CKD and acute kidney injury (AKI) compared to patients without IBD. This relationship was noted to be even stronger among young patients with the disease, who are known to have a higher rate of EIMs compared to adults[9-11]. This manuscript aims to review the current literature on renal complications of IBD, pathophysiological mechanisms, the diagnostic workup, and the treatment options for patients with these conditions.

IBD AND RISK OF CKD

Literature has documented that the presence of IBD may lead to the progression of CKD by multiple mechanisms. IBD has been shown to affect renal function by causing glomerular or renal tubular injury[12,13]. Glomerular insults and GN may lead to proteinuria and reduced glomerular filtration rate (GFR), while direct tubular injury can result in AKI or increased excretion of tubular proteins. Animal models of colitis induced with dextran sulfate sodium (DSS) have been extensively employed to investigate changes in renal function and structure. Chang *et al*[14], utilizing this model, observed a decrease in kidney size and weight following colitis induction. They also noted a reduction in type IV collagen, a constituent of the glomerular basement membrane's (GBM) supportive extracellular matrix, and deposition of type I and type V collagens in the renal interstitium. Damage to podocytes was also noted, particularly the loss of podocyte cytoskeletal proteins such as synaptopodin and podocalyxin. In a study by Ducasa *et al*[15], colitis induction by DSS resulted in increased bacterial translocation to the kidney and AKI, compared to controls.

The data regarding whether active IBD results in the progression of CKD is conflicting. Recent systematic reviews and large database studies have reported an increased risk of CKD in patients with IBD. In a retrospective study that included 10117 patients with IBD, it was noted that the hazard ratio of receiving a CKD diagnosis in patients with IBD was 1.24

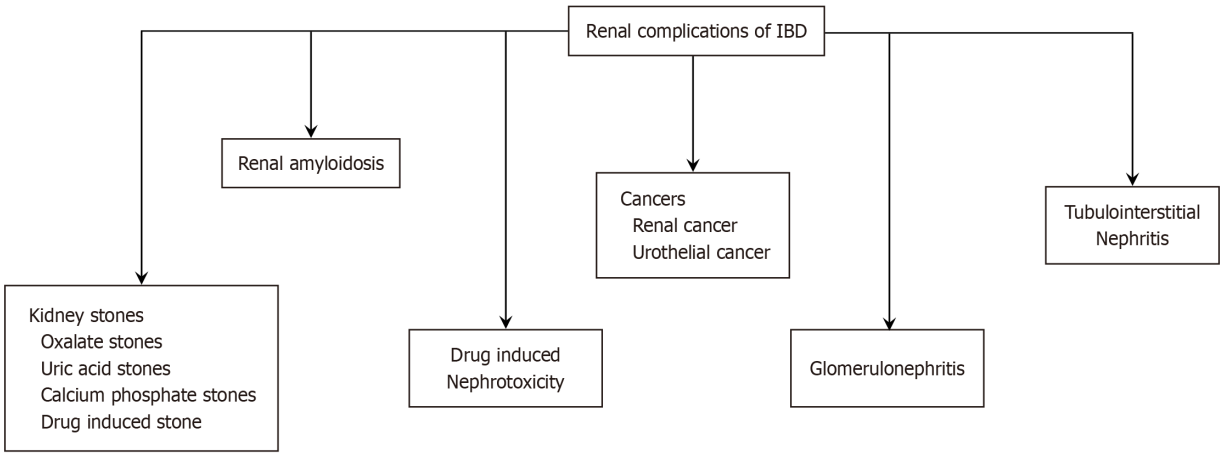

Figure 1 Common renal manifestation among patients with inflammatory bowel disease. IBD: Inflammatory bowel disease.

(95%CI: 1.10-1.40) compared to patients without IBD[16]. Another study with a similar design notes that the hazard ratio of AKI decreases with age, with the risk being 7.88 (95%CI: 2.56–24.19) at age 16 which decreased to 1.13 (95%CI: 1.01–1.25) at age 77[17]. Other studies have also demonstrated this finding of increased CKD risk in the younger population[18]. In a retrospective study involving children diagnosed with IBD, a non-significant trend of declining GFR was observed over six years since disease onset (113 mL/min/1.73 m² *vs* 103 mL/min/1.73 m², one-way ANOVA, P = 0.17)[19].

Kidney function can be clinically monitored by following creatinine levels or by proteinuria. Mahmud *et al*[20] in 1994 were the first to demonstrate that patients with active IBD exhibited significantly higher levels of microalbuminuria compared to those in remission (206 μg/min *vs* 65 μg/min, $P < 0.001$)[20]. Wu *et al*[21] utilized genome-wide associate study data to investigate the relationship between IBD and renal changes. They demonstrated a causal relation between ulcerative colitis (UC) and elevated albumin levels in urine in only one of their models (Inverse variance weighted method) but did observe a causal relationship between Crohn's disease (CD) and albuminuria. Similarly, in a prospective study involving 86 patients, Poulou *et al*[22] failed to demonstrate a difference in albuminuria between active and inactive IBD. Overall, the clinical evidence of microalbuminuria in IBD is conflicting and is of limited utility in clinical settings.

Reduction in GFR has been noted in patients with active IBD however the evidence is conflicting[23]. A retrospective review found IBD to be associated with an increased risk of CKD development; however, they failed to demonstrate a reduction in estimated GFR (eGFR) in patients with active IBD (−0.10 mL/min/1.73 m²; 95%CI: -0.33 to 0.48)[17]. For monitoring changes in GFR, cystatin C has been noted to be more sensitive than creatinine levels, particularly in patients with severe IBD[19]. We also hypothesize that associated factors such as an increased risk of stone formation, urinary tract infections (UTIs), and drug-induced nephrotoxicity may also play a more significant role in CKD development. Thus, regular monitoring of kidney function is generally recommended.

NEPHROLITHIASIS

Nephrolithiasis is one of the most common renal manifestations in IBD patients and has been described in case reports as old as the 1970s[24]. While the prevalence varies with different studies, ranging from 9%-28%, there is little doubt that stone formation is more common in patients with IBD compared to the general population[25-27]. The incidence of asymptomatic nephrolithiasis has been reported as high as 38% of IBD patients in some studies[28]. Among patients with IBD, there is a greater risk of stone formation in adults, males, patients using non-steroidal anti-inflammatory drugs and with lower levels of physical activity, patients with CD rather than UC, and higher in patients who have undergone surgical procedures, particularly terminal ileum resection or intestinal bypass[26-30]. Cury *et al*[28] also demonstrated that the extent of bowel disease, and ileocolonic involvement rather than just ileal or colonic involvement was also a relevant contributor.

Recurrent nephrolithiasis and the number of procedures it necessitates are both risk factors associated with the development of CKD in patients with IBD[26]. Additionally, compared to a patient without IBD, those who develop kidney stones with concomitant IBD are more prone to UTIs, sepsis, AKI, and end-organ failure and are more likely to need hospital admission[30]. Owing to their recurrent nature and requirement for repeated interventions, kidney stones in IBD patients are associated with increased morbidity[26]. The description of various stones among patients with IBD is presented below.

Calcium oxalate

The pathogenesis of oxalate stones in IBD is multimodal. Involvement of terminal ileum results in malabsorption of bile salts and fatty acids. This causes calcium to bind to fatty acids rather than forming calcium oxalate complexes, leading to increased intestinal absorption of oxalate with consequent “enteric hyperoxaluria”, defined as urinary oxalate concen-

tration > 45 mg/dL[31]. Additional proposed mechanisms include altered colonic mucosal permeability to oxalate and changes to the gut microbiome (Figure 2). The latter refers to the decolonization of *Oxalobacter formingens*, which reduces colonic oxalate catabolism[5]. The importance of this bacterium is supported by the finding that oral Oxalobacter administration is associated with reduced urinary concentrations of oxalate[32]. The role of hyperoxaluria may not be limited to oversaturation and precipitation. Studies have shown that oxalate proves toxic to the renal tubular epithelium and causes changes in gene expression, mitochondrial function, and cell death by oxidative damage, a series of events termed "oxalate nephropathy"[33]. Chronic exposure of tubular epithelium to oxalate can cause desensitization and thus impair protective countermeasures, predisposing patients to recurrent stone formation and CKD[34].

Uric acid stones

Diarrhea is common in IBD and leads to dehydration as well as loss of bicarbonate, leading to the excretion of concentrated, acidic urine which promotes the formation of uric acid stones[34]. Surgical procedures involving small bowel and colonic resection contribute[34]. This is accompanied by a reduced concentration of stone-inhibiting factors such as citrate and magnesium, all of which likely play a synergistic role in stone formation[26]. Serum or urinary levels of uric acid do not need to be increased to promote stone formation, rendering their monitoring unnecessary[35].

Calcium phosphate

Calcium phosphate stones are mainly seen in pediatric IBD patients, the pathogenesis of which is not well-understood [36]. It may represent increased calcium mobilization from the bone in the setting of fat-soluble Vitamin D malabsorption (Figure 2)[29].

Drug-induced nephrolithiasis

Treatment of IBD can also carry a risk of nephrolithiasis. There have been infrequent reports of sulfasalazine causing crystalluria and stone formation[26]. Vedolizumab, a monoclonal antibody directed against α4 β7 integrin is used in the treatment of moderate to severe IBD, especially UC[37]. A study found that vedolizumab use, as well as using two or more biologic drugs for treatment was associated with a greater risk of nephrolithiasis as compared to no biologic use. It is unclear if this is attributable to drug use or a consequence of more severe IBD in the patients who need this treatment [37]. However, it may be valid to consider stopping drugs that induce stone formation in these patients[38]. Discussion regarding the other nephrotoxic effects of drugs for IBD is presented below in a separate section.

Given the morbidity carried by nephrolithiasis in IBD, it is essential to adopt a meticulous approach to early diagnosis and treatment of the same. Diagnosis of nephrolithiasis can be challenging as the pain of nephrolithiasis can be confused with pain from an IBD flare. Low-dose computed tomography (CT) of kidneys, ureters, and bladder is mostly employed for diagnosis in the emergency setting[30]. Currently, guidelines do not advocate for routine urinary imaging[26]. However, IBD patients are still exposed to a high amount of radiation overall. Thus, current research is focusing on the possibility of using ultra-low radiation techniques for follow-up of IBD patients with nephrolithiasis[30].

Prevention and treatment are based on a mixture of dietary, medical, and surgical measures[26]. Dietary modifications include increased water intake to minimize dehydration from diarrhea and ostomies, increased citrate consumption, bile salt sequestrants, and pyridoxine supplementation[27]. Citrate therapy has been shown to prevent stone formation as well as help in stone expulsion[39]. It is also recommended that magnesium and citrate replacement should focus on correcting urinary levels of these substances instead of serum levels[30]. Consumption of oxalate and fat should be reduced especially in patients with CD and ileal resection[26]. Prevention of uric acid stones involves reducing dietary intake of purines, hydration, oral potassium citrate, and urine alkalinization[27]. Allopurinol and other xanthine oxidase inhibitors reduce uric acid levels in both serum and urine[30]. Treatment of stones ranges from conservative to extracorporeal shockwave lithotripsy and surgery, depending on the size of the stones and the presence of complications such as UTI and hydronephrosis.

RENAL AMYLOIDOSIS

CD has been reported to be the fourth leading cause of secondary amyloidosis, after chronic inflammatory arthropathy, chronic infections, and periodic fever syndromes[40,41]. Chronic immune-mediated inflammation in IBD leads to an increased risk of AA amyloidosis. Incidence is noted to be higher in patients with CD (0.3%-10.9%) compared to UC (0%-0.7%)[42-45]. This is usually attributed to the fact that there is a wider extension of inflammation in CD compared to the UC[46,47]. In one study it was noted that 54% of the cases with amyloidosis secondary to IBD had extensive ileocolonic involvement[48]. Interestingly it was also noted that the development of amyloidosis was twice as prevalent in men as compared to women diagnosed with IBD[48]. Amyloidosis typically occurs as a longstanding sequelae of IBD, however simultaneous diagnosis is not uncommon due to diagnostic delays[45,49,50].

Renal amyloidosis in IBD usually presents proteinuria in the setting of nephrotic syndrome, eventually leading to renal impairment. However, approximately 15% of the cases, have neither at presentation, and thus a high index of suspicion is required[48]. In many IBD patients, amyloidosis can also present as malabsorption which is not explained by the underlying disease activity[48]. Renal biopsy is the gold standard test for diagnosis. A high majority of cases have both mesangium and glomerular involvement. In many cases when the suspicion of renal amyloidosis is high but renal biopsy is difficult to obtain, abdominal fat pad or rectal biopsies can be done.

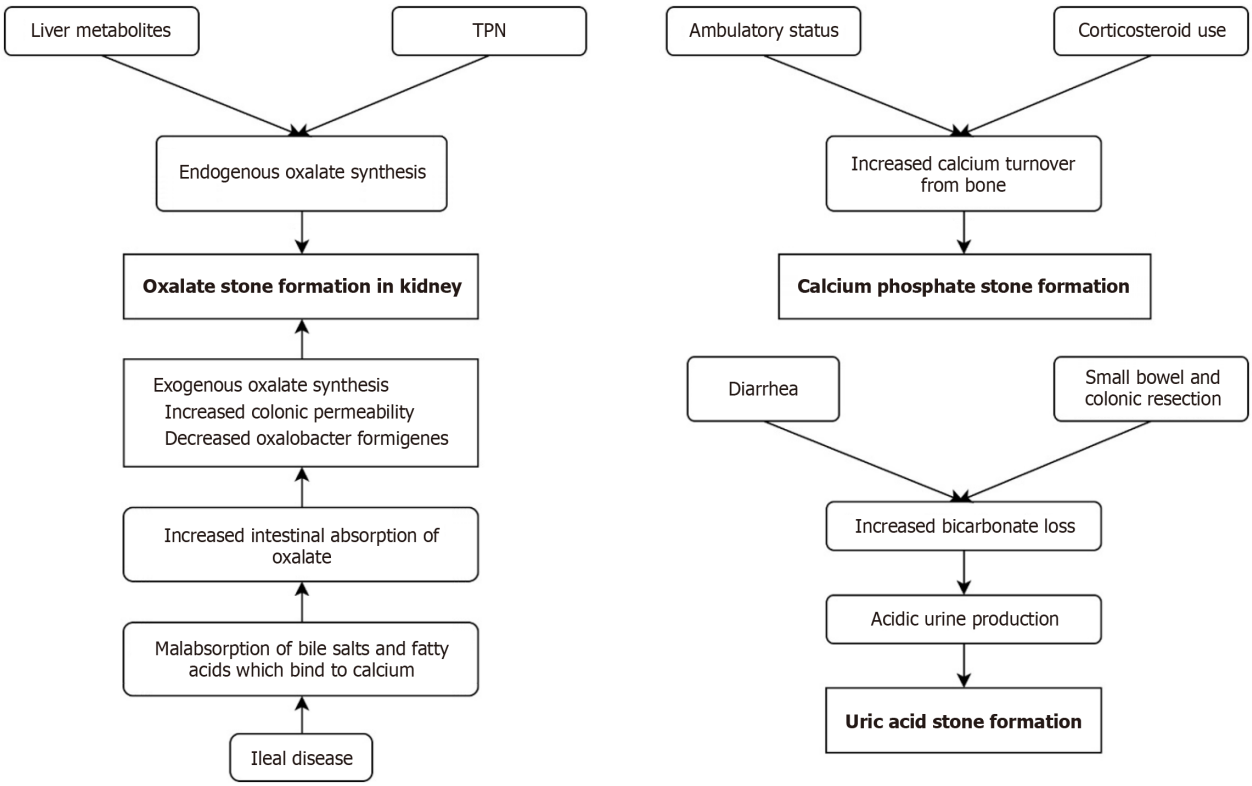

Figure 2 Pathogenesis of stones among patients with inflammatory bowel disease. TPN: Total parenteral nutrition.

Since renal amyloidosis is a rare EIM, there is a lack of therapeutic studies and thus the effectiveness of treatment is not established[42,43,51]. The primary objective of treatment is to control the underlying inflammatory state, decrease the formation and deposition of circulating AA protein, and reverse the deposits already present in the affected organs[49]. Immunosuppressive drugs (methotrexate, cyclosporine, and azathioprine), colchicine, dimethylsulfoxide, and corticosteroid have all been, without established benefits. Initially, colchicine was proposed as an effective therapy, based on the favorable response seen in cases of amyloidosis secondary to periodic fever syndromes[52].

Even if started early on colchicine, proteinuria was observed to have been reduced and renal function would remain stable over a while but no benefit was observed in the reduction of amyloid depositions[53-58]. Favorable outcomes have also been noted with anti-TNF drugs such as infliximab and adalimumab[44,59-62]. Combining anti-TNF, immunosuppressants, and colchicine could improve prognosis. Many studies have also reported clinical improvement of IBD-related amyloidosis after surgical resection of the involved intestinal segment[63-65]. The suggested mechanism behind it is the decrease in the production of serum amyloid A after the surgical resection of the affected organ. Newer treatment options include drugs targeting interleukin (IL)-6 such as tocilizumab or drugs targetting serum amyloid P component such as (R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid and therapeutic IgG anti-SAP antibodies[66-69].

Even with the recent biochemical advancements the treatment needs to be individualized as there is a lack of understanding of the long-term efficacy and safety of biological agents in clinical settings. In addition to management of the IBD, medical management of renal injury is also imperative in these patients. Therapy for nephrotic syndrome is symptomatic with restriction of sodium intake, use of angiotensin-converting enzyme inhibitors, and statin may be needed in certain scenarios. Monitoring proteinuria and serum amyloid A protein can be useful to assess the response to treatment and prognosis. Many patients may end up with end-stage renal disease requiring dialysis and/or transplantation.

RENAL AND UROTHELIAL CANCERS IN IBD

Extraintestinal cancers are significant concerns for individuals with IBD. However, there's a lack of research on the link between IBD and urothelial cancer. In individuals with IBD, there exists a significant overrepresentation of malignancies originating in the kidneys and urinary tract with the relative risk being five times higher compared to the general population[70,71]. Although the mechanism underlying the relationship between IBD and oncogenesis is not fully understood, there is evidence suggesting that inflammation not only serves as the body's response to malignant tumors but also plays a role in triggering carcinogenesis[72]. Additionally, immunosuppressive medications frequently utilized in the treatment of IBD are believed to be associated with a heightened risk of various malignancies, including non-Hodgkin lymphoma, acute myeloid leukemias, non-melanoma skin cancers, and urinary tract cancers[73].

A recent meta-analysis of 9 cohort studies indicated a significantly elevated risk of renal cancer in individuals with IBD, particularly those diagnosed with CD. In contrast, no significant increase in risk was observed among patients with UC when compared to the general population[74]. Previous epidemiological investigations have established a robust association between IBD and an increased risk of various cancers[75]. However, conclusive evidence supporting a direct link between IBD and bladder cancer risk remains elusive. Kappelman *et al*[76] conducted a comprehensive study spanning over 30 years and reported a slight increase in the risk of bladder cancer among patients with CD [standardized incidence rate (SIR): 1.1; 95%CI: 0.8 to 1.6].

In patients diagnosed with CD, a significant correlation has been established between cigarette smoking and urological malignancies. However, such an association has not been consistently observed in individuals with UC. In a study conducted by Madanchi *et al*[77], which analyzed the incidence of malignancies in IBD centers over 7 years, a higher occurrence of bladder cancer was observed in IBD patients compared to those without IBD (21.7 per 100000). Conversely, Algaba *et al*[70], in a cohort study, did not identify a significant overall increase in the risk of cancer among individuals with IBD. However, they did find that the risk of bladder cancer remained elevated (RR: 5.23; 95%CI: 1.95–13.87). Both studies by Madanchi *et al*[77] and Algaba *et al*[70] are single-center cohort studies indicating a statistically significant increase in urothelial cancer among IBD patients. However, the sample sizes differ (1026 *vs* 590 patients), potentially introducing bias due to the smaller cohorts. Conversely, Pedersen *et al*[78] observed no difference in the risk of bladder cancer between patients with IBD and the general population (SIR: 0.99; 95%CI: 0.63–1.54). In a study encompassing nearly 19500 patients diagnosed with IBD, 16 individuals developed urological malignancies. Upon conducting a multivariate analysis, the use of thiopurines was found to be associated with a threefold increased risk of urinary tract cancers[79]. Further investigation through larger case-control studies is essential to comprehensively comprehend the underlying mechanisms and refine clinical approaches for renal cancer surveillance in IBD patients. Additionally, there remains a significant gap in understanding the association between IBD and urinary tract malignancies, necessitating continued research efforts to address unanswered questions in this area.

GN

GN is a relatively infrequent complication of IBD but has been extensively studied. These include IgA nephropathy (IgAN), membranoproliferative GN, membranous glomerulopathy, minimal change disease (MCD), C3 glomerulopathy, and anti-GBM GN[26,80]. Of these, IgAN is the most prevalent, and reports of the association date back to 1984[26,80]. A study of renal biopsies in IBD patients with acute or chronic renal failure demonstrated IgAN in a majority of the samples [81].

The pathogenesis of GN in IBD is poorly understood and different theories have been proposed. One theory is that IBD and IgAN share genetic predisposition. Both have shown an association with HLA-DR1[82]. Novel genetic loci have also been identified in IgAN recently. These include CARD9, VAV, and PSMB 8/9, genes involved in the activation of NF-kB, maintaining the gut mucosal barrier, and regulating local inflammation[83,84]. Additional regions include TNFSF13 and DEFA[84,85]. DEFA encodes alpha-defensins, a type of antimicrobial formed by Paneth cells in the small intestine. Reduced levels of alpha-defensins have been reported in CD patients[86]. Inflammation from cytokines could contribute to both IBD and glomerular damage. Complement activity also plays a role in both diseases. It is known to promote glomerular sclerosis and interstitial fibrosis in IgAN[87]. Activated C3b has also been seen in intestinal mucosa and resected ileocecal specimens in patients with CD[88]. Mucosal inflammation in IBD alters gut mucosal permeability leading to antigen exposure, formation of autoantibodies, and consequent deposition of antigen-antibody complexes[35]. Considering that IgA acts as a mucosal antibody, it is reasonable that gut mucosal inflammation would be associated with IgA dysregulation. IgAN patients have been known to possess antibodies to dietary antigens, which have been recovered from sera as well as immune complexes deposited in the glomeruli[89]. A similar pathogenesis was first suggested for the association of IBD with celiac disease[90] Ileostomy and colostomy have also been associated with IgAN, which may be attributable to exposure of skin flora to the gut and the ensuing immune response[91]. The gut microbiome can also produce B cells activating TNF factors and overstimulate B cells, which causes a shift from IgA2 to IgA1 generation[92, 93]. Moreover, TNFSF13, identified as a susceptibility locus for IgAN encodes APRIL, a TNF ligand involved in B-cell maturation, response to mucosal antigens, and IgA production in gut-associated lymphoid tissue[91]. Studies have also shown aberrant O-linked glycosylation of IgA in CD patients[94]. Additionally, the role of T-cells has also been questioned, given the importance of costimulation in gut immunity. In animal models of IgAN, T-cells have been observed to play a critical role in controlling intestinal inflammation[95]. MCD development might also be associated with IBD treatment[35].

Another possibility is that GN is simply a concurrent EIM of IBD. This is supported by the frequent appearance of GN with IBD flares and improvement after treatment of gut disease[35]. Overall, it is unclear if IgAN occurs as a consequence of IBD flares exclusively or develops separately as individuals prone to IBD likely have a genetic makeup that predisposes to IgAN. Aside from IgAN, the pathophysiology behind other forms of GN is less clear[27].

Diagnosis of IgAN is mostly suspected based on occult blood and/or proteinuria on urinalysis and confirmed on biopsy[91]. Elevated serum IgA levels have been noted but are not a consistent finding[81]. Patients who are suspected to have nephropathy based on labs but not referred for biopsy are labeled as "suspected IgAN"[91]. Treatment of IBD-associated IgAN mainly focuses on treating the IBD, mostly with steroids[36]. In most cases, this improves renal function. Clearance of IgA deposits as well as mesangial proliferation has been observed on follow-up biopsy after treating IBD, suggesting sufficient healing[96]. A course of enteric budesonide has resulted in improvements in proteinuria and normalization of kidney function, further supporting this theory[97,98].

It is currently unclear if concomitant IBD forbodes a poor prognosis in IgAN. Some studies have reported similar rates of kidney failure in IgAN with and without IBD, but some have described a renal failure rate of up to 50% in IBD-related IgAN[99,100]. This might be explained by shorter follow-ups in some studies, which could miss the development of kidney failure[80]. A study found that CD-associated IgAN patients were more likely to experience aggressive IgAN, with glomerulosclerosis, extensive tubular atrophy, and interstitial fibrosis[84]. It thus appears reasonable to maintain a high index of suspicion for IgAN in IBD patients and follow these patients closely to prevent potentially adverse outcomes.

Other patterns of glomerulopathy are less prevalent but have been reported in IBD. An Egyptian study reported that crescentic GN is the most common pattern after IgAN[101]. It was associated with the presence of ASCA, which tends to present in more severe forms of IBD. Moreover, it showed associations with p-ANCA and c-ANCA, possibly indicating that ANCA found in IBD patients might be targeting tissues similar to those in ANCA-associated renal vasculitis[101]. A case report described a 75-year-old patient with UC who presented with symptoms of an IBD flare and worsening renal function. A renal biopsy revealed C3 glomerulopathy. The mechanism is uncertain, but complement activation and deposition are presumed to play a critical role in UC and may be responsible for glomerulopathy as well[102]. Another study from China found membrane glomerulopathy to be the most prevalent pattern after IgAN[103]. Genetic associations between IBD and glomeruli nephropathies other than IgAN have not been found yet[28]. Further research is needed to assess the molecular mechanisms responsible for these associations.

TUBULOINTERSTITIAL NEPHRITIS

In a retrospective review of renal biopsy specimens among patients with IBD and renal injury, interstitial nephritis was noted to be the second most common diagnosis after IgAN[81]. Tubulointerstitial injury has been noted to occur secondary to systemic inflammatory and immunological reactions, however, there is still no consensus in regards to the exact mechanism. In animal models, renal tubular injury has been associated with neutrophil infiltration and the expression of cytokines and chemokines, resembling the pathophysiology of colitis characterized by neutrophil infiltration in the colonic epithelium. This is proposed to be mediated *via* keratinocyte chemoattractant (KC) receptors[104]. Ranganathan *et al*[105] demonstrated that genetic deletion of CXCR2, a type of KC receptor, led to the suppression of AKI in animal models. In a similar study design, overexpression of Netrin-1, a chemotropic cue with anti-inflammatory properties, in proximal tubular epithelial cells suppressed cytokine expression and neutrophil infiltration[13].

In addition, 5-aminosalicylates (ASAs), cyclosporine A (CsA), and TNF-α inhibitor exposure are known to cause tubulointerstitial nephritis. As 5-ASA compounds have shown good efficacy in maintaining disease remission, frequent and long-term use of these compounds is common. One study noted serious renal impairment in 1 of 500 patients treated with 5-ASA derivatives[106,107]. Both direct injury secondary to inflammation and medication can independently cause tubulointerstitial damage. Larchet *et al*[108] were the first to describe an association of interstitial nephritis with IBD in an adolescent population. Since then, numerous studies have reported the incidence of nephritis even in the early stages of the disease.

Clinically, these changes manifest as increased urinary levels of tubular proteins such as N-acetyl-B-D-glucosaminidase (NAG), α-1-microglobulin, and beta-2-microglobulin. Fraser *et al*[109] noted that over 50% of patients with new diagnoses of IBD had increased levels of NAG and α-1-microglobulin. However, no correlation was found between protein levels and disease activity, and treatment did not significantly alter these levels[109,110]. Thus, apart from demonstrating kidney injury, their clinical significance for screening or indicating a decrease in disease activity has not been documented.

The association between TIN in IBD patients and drug therapy is bolstered by those reports demonstrating a strong temporal relationship to drug exposure, recovery of renal function after withdrawal of the drug, and recurrence of renal injury upon rechallenge[111-113]. In cases of TIN secondary 5-ASA, complete recovery of renal function has been reported if TIN is diagnosed within 10 months from the start of treatment. The treatment for drug-induced AIN is the discontinuation of the offending drug and steroid therapy[114,115]. If diagnosis is delayed beyond 18 months, only one-third of cases show any recovery of renal function[107]. Unfortunately, many of the cases of TIN with exposure to nephrotoxic agents and concomitant IBD end up with ESRD within 3 years of diagnosis[116]. Therefore, for patients on regular 5-ASA and mesalamine therapy, it is now recommended that renal function should be assessed before initiation of medical therapy with repeat testing every 3 months for a year and then twice every year[107,115,117].

DRUGS AND RENAL TOXICITY

Drug-induced kidney damage is a prevalent issue in clinical practice, with drug-related AKI occurring in up to 60% of cases[118,119]. This condition often necessitates extensive and costly interventions, potentially including hospitalization [120]. It is crucial to note that all parts of the kidney can be impacted, leading to the development of classic clinical renal syndromes such as AKI, tubulopathies, proteinuric renal disease, and CKD (Table 1)[121]. The conservative management of IBD typically involves a combination of medications, including ASAs, steroids, antibiotics, immunosuppressants, and biologic agents. While the nephrotoxic effects of ASAs and CsA are well recognized, recent evidence suggests a potential role of biologic agents such as infliximab and adalimumab in contributing to renal impairment[122].

Table 1 Potential nephrotoxic effects of pharmaceuticals used in the treatment of inflammatory bowel disease

Pharmaceutical treatment	Potential nephrotoxic effects
Sulfasalazine	Tubulointerstitial nephritis, gloermurlonephritis
CsA	Nephritis, interstitial fibrosis
TNFα Inhibitors	Glomerulonephritis
Methotrexate	None at conventional doses
Azathioprine	No direct evidence of a renal effect
Corticosteroids	None
Antibiotics (*e.g.*, ciprofloxacin)	Acute kidney injury
Vedolizumab	Acute interstitial nephritis
Tofacitinib	Acute kidney injury
Filgotinib	Increased drug concentration in renal impairment

CsA: Cyclosporine A.

ASAs

5-ASAs are the primary treatment for IBD. Different formulations have been developed to enhance 5-ASA absorption in the inflamed intestine. Both sulfasalazine (5-ASA bound to sulfapyridine) and coated 5-ASA (mesalazine, olsalazine) have been linked to renal toxicity[123]. Research indicates that 5-ASA administration can directly impact renal function, with renal impairment occurring in up to 1 in 100 patients, but clinically significant damage affecting only 1 in 500 patients [107,124]. Renal toxicity due to 5-ASA may present as GN, minimal-change nephropathy with nephrotic syndrome, and interstitial nephritis, which may be associated with nephrogenic diabetes insipidus. Several instances of renal toxicity have been documented with sulfasalazine, presenting as an idiosyncratic, dose-independent occurrence within a broader hypersensitivity reaction[125]. Conversely, mesalazine has been associated with a notable incidence of nephritis development in patients during its use. Mesalazine has the potential to induce acute or chronic interstitial nephritis[115]. Salicylate is filtered and actively secreted in the kidney *via* proximal tubules, with subsequent passive reabsorption leading to elevated cortical and medullary concentrations. Inhibition of intrarenal prostaglandin synthesis by salicylates disrupts intrarenal blood flow regulation and mitochondrial oxidative phosphorylation, potentially causing regional hypoxia. Moreover, high intrarenal salicylate levels inhibit the pentose phosphate shunt, reducing renal glutathione and making the kidney vulnerable to oxidative damage.

Cyclosporine

Low-dose CsA administration showed no significant nephrotoxicity, while high-dose CsA led to renal impairment in 6% of patients[126,127]. Overall, patients receiving CsA treatment for autoimmune diseases, including IBD, experience a 20% reduction in their GFR[128]. CsA can induce acute renal dysfunction by causing significant vasoconstriction of the afferent arterioles. This constriction leads to a decrease in renal blood flow and GFR, accompanied by elevated serum creatinine levels. Typically, renal function improves within 5 to 7 days after reducing or discontinuing CsA treatment [129]. CsA can lead to chronic renal impairment, although the precise mechanism of this nephrotoxicity is not fully understood. However, it has been suggested that the activation of the renin-angiotensin system within the kidney may play a significant role in the development of chronic CsA nephrotoxicity[130]. The recommended duration of CsA therapy should not surpass 4–6 months, after which an alternative remission maintenance drug should be considered. CsA dosages should be lowered if baseline serum creatinine levels increase by more than 30%[131]. It's important to avoid concurrent use of other nephrotoxic agents, and patients with preexisting renal dysfunction should likely not be treated with CsA[127].

TNF-α inhibitors

While TNF-α inhibitors, specifically infliximab and adalimumab, have demonstrated significant efficacy in inducing and maintaining clinical remission in IBD, they are associated with serious adverse effects. Despite earlier reports indicating the potential benefits of TNF-α inhibitors in the treatment of GN, recent studies and increased experience with these inhibitors have raised questions about their efficacy in GN and highlighted a potential role in the development of renal complications, including GN. Although data on GN in IBD patients due to TNF-α inhibitor therapy are limited, recent reports suggest a potential triggering effect of TNF-α inhibitors on renal impairment, indicating a significant adverse effect[132]. One possible mechanism for the development of renal complications during anti-TNF-α administration involves the interaction of anti-TNF-α antibodies with TNF-α present in glomerular visceral epithelial cells[133].

Nephrotoxicity induced by other therapeutic agents in IBD

Vedolizumab, a humanized monoclonal antibody targeting α4β7 integrin and primarily acting in the gut, is utilized to

manage UC and CD[134]. The therapy has demonstrated efficacy in both initiating and sustaining remission in IBD. It is generally well received and is perceived to possess a favorable safety profile compared to alternative 'biologics' utilized in managing IBD[135]. Recent case studies propose that vedolizumab might contribute to the development of acute interstitial nephritis. Reported cases have emerged indicating occurrences of interstitial nephritis believed to be linked to the use of vedolizumab. This underscores the necessity for ongoing awareness regarding rare adverse drug reactions, even in treatments that are typically well-tolerated.

Tofacitinib is an orally administered medication, that acts as a partially selective inhibitor of Janus kinase (JAK). This small molecule functions intracellularly to impede JAK-dependent cytokine signaling. By blocking these enzymes, tofacitinib helps regulate immune and inflammatory reactions[136]. Fixed-dose regimens of tofacitinib achieved improved kidney function and showed comparable effectiveness to CsA in kidney transplant patients. However, this came with a higher likelihood of experiencing certain adverse events[137].

Filgotinib is a medication taken by mouth, designed as a preferential inhibitor of JAK1. By primarily targeting JAK1, it influences a specific set of proinflammatory cytokines within the JAK-signal transducer and activator of the transcription pathway. This subset differs from those affected by inhibition of JAK2 or JAK3. Pharmacokinetic investigations have revealed elevated drug concentrations in individuals with an eGFR < 60 mL/min/1.73 m². Therefore, it is recommended to consider dose reduction for such patients. Filgotinib hasn't been studied in patients with end-stage renal disease (eGFR < 15 mL/min/1.73 m²)[138]. As a result, its use is not recommended in this population. Ustekinumab, an anti-IL-23 biologic, presents an alternative treatment for individuals with moderate to severe UC and CD. However, it is important to note that its use may be linked to nephrotic syndrome arising from focal segmental glomerulosclerosis[139].

Other therapies utilized in IBD therapy, include corticosteroids, thiopurines (azathioprine and 6-mercaptopurine), methotrexate, and mycophenolate mofetil, which have shown no direct significant effects on renal function. Specifically, methotrexate, despite the potential for nephrotoxicity at high doses, does not exhibit nephrotoxic effects at conventional doses typically used in IBD treatment[140]. Concerning azathioprine, there is no evidence indicating a direct renal effect. The prolonged use of antibiotics like ciprofloxacin and metronidazole, as well as the use of total parenteral or enteral nutrition, does not seem to induce renal impairment. However, there have been a few case reports suggesting nephrotoxic effects associated with ciprofloxacin[141]. Furthermore, there is abundant data regarding the role of corticosteroids on kidney function which is beyond the scope of this article. Distinguishing extraintestinal renal dysfunction from drug-induced injury is complex and vigilant renal function monitoring is required to diagnose drug-induced nephrotoxicity as prompt cessation of toxic medications is vital to prevent further harm and potentially reverse renal injury.

FISTULAS AND HYDRONEPHROSIS

CD is characterized by sustained transmural inflammation of the bowel wall leading to fistula formation which can be noted in about one-third of the patients with CD[142]. The inflamed intestine can adhere to the bladder wall leading to the formation of an enterovesical fistula (EVF). CD is the third most common cause of EVF behind diverticular disease and malignancy[143]. The incidence of EVF is rare with a reported incidence of 2%-5% among patients with CD, with ileovesical fistula being the most common (64.9%)[144]. Males are noted to be affected by EVFs at a higher rate than women in most of the major studies, and this has been attributed to the anatomical barriers imposed by the uterus and vagina[144-146].

Clinically EVF is characterized by pneumaturia, fecaluria, recurrent or persistent UTIs, and urorrhea. Recurrent UTIs in a patient with CD justify a diagnostic workup for a urinary system fistula[147-149]. Enteric bacteria, Escherichia coli are the usual infective agents. Historically, poppy seed tests, plain abdominal X-rays, and barium enema were used, but these have been superseded by cross-sectional imaging[150,151]. Although not routinely recommended, Cystoscopy should be used in patients with EVF secondary to malignancy to rule out bladder involvement[152]. It can also be used to exclude other etiologies such as bladder stones and interstitial cystitis[153].

An abdominopelvic CT with oral or rectal contrast (but not IV contrast) is the imaging test of choice for diagnosing EVF and some authors have reported that CT can accurately detect the presence of EVF in up to 90 to 100 percent of patients even though direct visualization of the tract is limited[143]. Suggestive CT findings include intravesical contrast or air without prior instrumentation. Radiation dose is one of the major deterrents against CT scan and most of the CD patients are young and would require multiple scans for evaluation over the years. Magnetic resonance imaging allows the accurate depiction of fistulous tracts with the advantage of being radiation-free. T2-weighted sequences and intravenous contrast are widely used to delineate fistula anatomy and identification of associated abscess collections in patients with CD. However, whether magnetic resonance imaging is superior to CT for the detection of EVF remains controversial, and further prospective studies are needed[154]. Endoscopy has a very low sensitivity for detecting a fistulous tract but can be used to determine the underlying etiology of the fistula.

Initial management depends upon whether symptoms are present at the time of presentation. Asymptomatic patients can be managed just with medical therapy to control CD. Studies have shown that long-term remission can be achieved in up to 35% of patients[144,155,156]. Antibiotics are necessary in patients who are initially present with infections. Medical management is also recommended in patients who are not suitable for surgery due to a poor condition, intolerance to anesthesia, or terminal disease. Despite the potential promise of medical treatment, most of the patients with EVF require some form of surgical management. Retrospective reviews have noted that around 80%-85% of the patients needed surgical management[157]. Taxonera *et al*[144] noted that 78 of the 79 patients who underwent surgical management noted improvement in the symptoms. Although an open approach is preferred in most cases, the laparoscopic approach and bladder-preserving procedures can be considered in some limited presentations.

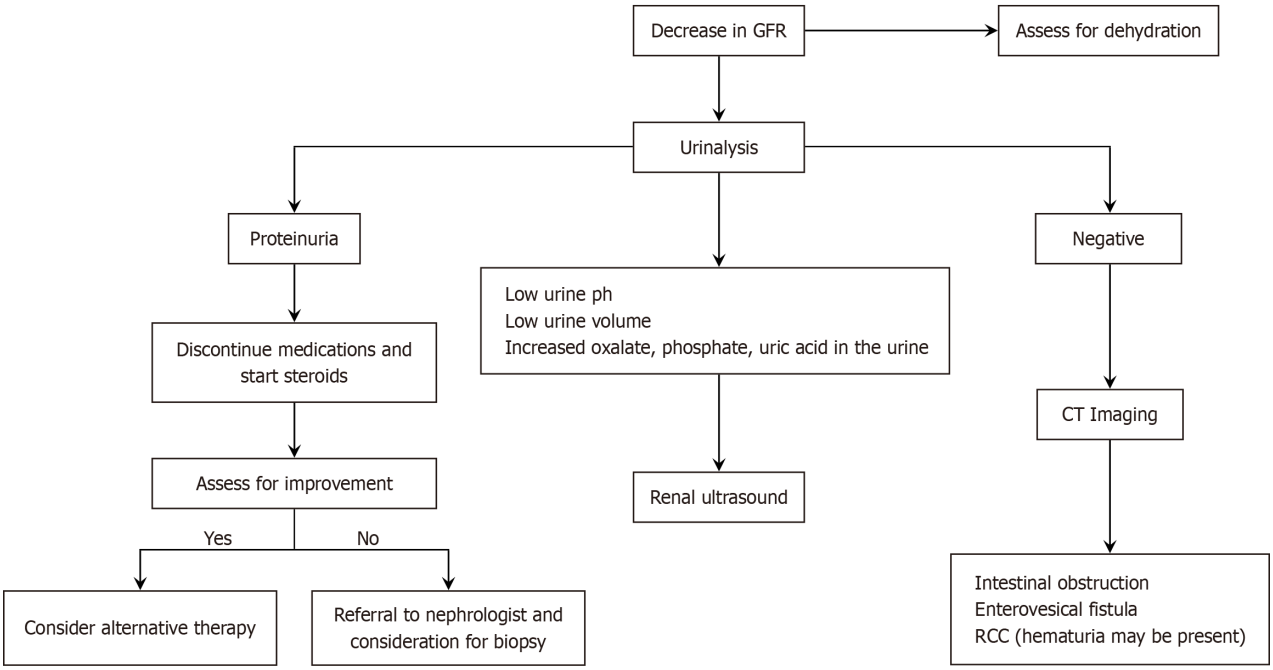

Figure 3 A proposed clinical strategy for the workup of inflammatory bowel disease patients with decreased glomerular filtration rate. CT: Computed tomography; GFR: Glomerular filtration rate; RCC: Renal cell carcinoma.

Hyams *et al*[158] in 1943, presented the first documented case of obstruction of the ureter and kidney associated with CD. Hydronephrosis is noted to occur when inflammation, fistulas, abscess formation, or fibrosis occurs in the bowel adjacent to the ureters. Obstruction is usually noted on the right side at the level of the linea terminalis (75%-100%)[159-163]. Hydronephrosis in CD can also occur secondary to a renal stone obstructing the ureteropelvic junction[164].

Overall the incidence of hydronephrosis varies from around 3.1% to 6%[160,165-167]. In a retrospective review, 4 of 62 (6%) of the patients with CD were noted to have hydronephrosis. Three of them had right-side hydronephrosis with the mean duration between the onset of CD and diagnosis of hydronephrosis being 5.6 years[160].

The primary goal of managing hydronephrosis in CD is ureteral drainage to prevent damage to the obstructed kidney. Some minimally invasive options include percutaneous nephrostomy or indwelling ureteral stenting. This is done alongside medical therapy. Ben-Ami *et al*[160] noted that 3 out of the 4 patients showed a good response to pulse steroids while Angelberger *et al*[161] noted success in one patient. The surgical approach is usually the last-line modality and involves resection of the affected bowel along with/without ureterolysis[167].

MONITORING OF KIDNEY FUNCTION FOR IBD PATIENTS

In terms of kidney function monitoring, there is a general agreement on the validity of using serum creatinine levels alongside estimating GFR using either the MDRD or CKD-EPI equations. Additionally, there is widespread acknowledgment of the importance of including a blood ionogram and analyzing urine samples for the protein-to-creatinine ratio. Recommendations are made to integrate these monitoring practices at the onset of IBD diagnosis, before introducing new treatments, and annually for screening extraintestinal manifestations (EIMs) and assessing treatment tolerance. Specifically, it is suggested to evaluate kidney function three months post-initiation of mesalamine therapy, followed by assessments every six months, while annual monitoring is considered adequate for patients receiving biologics[168].

CONCLUSION

Among patients with IBD, the renal and urological complications are difficult to diagnose as the associated symptoms in these patients can be subtle. Thus, a high index of suspicion is warranted to diagnose these early as just like other EIMs, the presence of renal manifestations can lead to reduced quality of life. Currently, there is a lack of international guidelines regarding the standardized timing of kidney function monitoring. Furthermore, there is no consensus regarding how to approach AKI among patients with IBD. We provide a diagnostic workup that may be beneficial in managing patients with decreased GFR among patients with IBD (Figure 3). Patients with IBD are also at risk of repetitive kidney injury due to episodes of dehydration and various causes discussed above. Thus, a multidisciplinary discussion between gastroenterologists and nephrologists will be beneficial in preventing worse outcomes among these patients.

FOOTNOTES

Author contributions: Singh A, Sohal A, and Yang J conceptualized and designed the study. Singh A, Khanna T, Mahendru D, and Kahlon J conducted the literature review, interpreted data, created artwork, and drafted the original manuscript. Kuman V, Sohal A, and Yang J supervised the study and made critical revisions.

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MINIREVIEWS

Challenges in predictive modelling of chronic kidney disease: A narrative review

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Abstract

The exponential rise in the burden of chronic kidney disease (CKD) worldwide has put enormous pressure on the economy. Predictive modeling of CKD can ease this burden by predicting the future disease occurrence ahead of its onset. There are various regression methods for predictive modeling based on the distribution of the outcome variable. However, the accuracy of the predictive model depends on how well the model is developed by taking into account the goodness of fit, choice of covariates, handling of covariates measured on a continuous scale, handling of categorical covariates, and number of outcome events per predictor parameter or sample size. Optimal performance of a predictive model on an independent cohort is desired. However, there are several challenges in the predictive modeling of CKD. Disease-specific methodological challenges hinder the development of a predictive model that is cost-effective and universally applicable to predict CKD onset. In this review, we discuss the advantages and challenges of various regression models available for predictive modeling and highlight those best for future CKD prediction.

Key Words: Chronic kidney disease; Predictive modelling; Regression; Statistical modelling; Methodology

Core Tip: The burden of chronic kidney disease (CKD) is growing rapidly and there is an urgent need to prevent the growth of the disease burden by identifying the individuals at high risk for the development of CKD. A broad spectrum of statistical models exist that can predict the future onset of the disease. This narrative review discusses the practical applicability of various statistical models for CKD prediction.

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INTRODUCTION

The growing burden of chronic diseases calls for advanced preventive measures, proper screening, and early diagnosis to limit the economic burden. Preventive strategies through changes in lifestyle and dietary habits could limit the burden of chronic diseases. However, it is difficult to inculcate these changes, and it is a long-term process to reach targets of sustainable development goals to reduce premature mortality. Statistical methods could be effectively applied to predict the onset of these chronic conditions through well-developed and validated predictive models. Different predictive models have been developed for different chronic diseases[1,2]. However, the feasibility of applying existing models in real life with predictive accuracy and translational significance is still a major challenge among practitioners. Regional and sociodemographic differences of the individuals pose generalizability issues of the existing models. In addition, the appropriate modeling techniques, including model development and validation methods, are among the few other challenges for the practical application of the existing models.

Chronic kidney disease (CKD), among the broad spectrum of chronic diseases is on an exponential rise. Kidney Disease Improving Global Outcomes (KDIGO) guidelines define CKD as structural or functional abnormalities in the kidneys, present for > 3 mo[3]. Functional abnormalities in the kidneys can be assessed using glomerular filtration rate (GFR), which measures the rate of filtration of blood through glomeruli (network of blood vessels in kidneys). It is measured by clearance of exogenous filtration markers[4]. However, functional abnormalities in clinical practice are approximated by using estimated glomerular filtration rate (eGFR)[5]. It is calculated using serum creatinine or serum cystatin (endogenous markers) and classifies kidney function into G1–G5 categories, whereas KDIGO classification based on urine albumin-to-creatinine ratio (ACR) classifies the disease into A1–A3 categories. However, early diagnosis of CKD between stages 1 and 3 is challenging, as CKD remains asymptomatic in its early stage. Noninvasive markers show up when the majority of kidney tissue is already damaged. Thus, predictive modeling can address the issue and help ease the future CKD burden by predicting disease onset.

Several regression methods exist for the predictive modeling of the disease. The choice of the method depends on the distribution of the outcome variable and its relationship with the covariates. Nevertheless, each of the available regression methods is defined under a set of assumptions that are specific to the method under consideration. However, the extent to which the real data deviate from the defined set of assumptions poses a real challenge for statisticians. Internal validation, calibration and discrimination of the model have been suggested to be adequately considered when developing the predictive model of a disease[6,7]. The broad classification of the regression methods, based on the distribution of the outcome variable includes multiple linear regression, quantile regression, logistic regression, Poisson regression, and negative binomial regression (Figures 1 and 2). This review discusses the challenges associated with the application of these regression methods for the predictive modeling of CKD.

REGRESSION MODELS BASED ON CONTINUOUS OUTCOME VARIABLES

Simple/multiple linear regression model for CKD

Simple linear regression is the most basic regression method initially conceptualized and applied by Sir Francis Galton to solve the problem of heredity in the 19th century. The mathematical notation of the simple linear regression model is given by: $E(Y|X) = \mu(X) = \beta_0 + \beta_1(X)$, which is a line with intercept β_0 and slope β_1, with Y the outcome variable measured on a continuous scale and X the covariate.

Simple linear regression can be extended to multiple linear regression to include more than one independent variable, to model multifactorial diseases like CKD. Multiple linear regression analysis uses the ordinary least square estimation method to study the causal association between the outcome variable and the covariates[8]. Linear regression analysis relies on the basic assumption of the linear relationship between the predictor variables and the outcome; the outcome variable being measured on a continuous scale. However, as KDIGO classifies kidney disease based on eGFR and ACR categories, the application of linear regression to predict future kidney disease is irrelevant for the case of CKD. However, multiple linear regression can only be used to model changes in eGFR or ACR, which are continuous variables and also surrogate points for CKD[9]. Nevertheless, longitudinal cohort studies with longer follow-up periods are required to achieve the minimum sample size for the clinically significant decline in eGFR[10]. However, the assumption of the linear

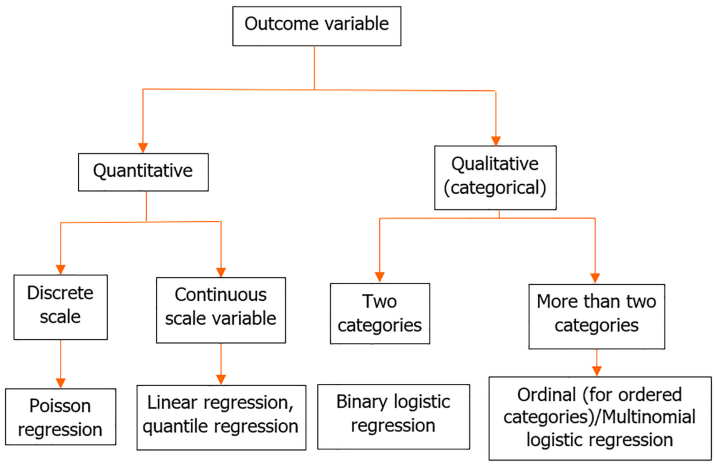

Figure 1 Selection of appropriate regression model.

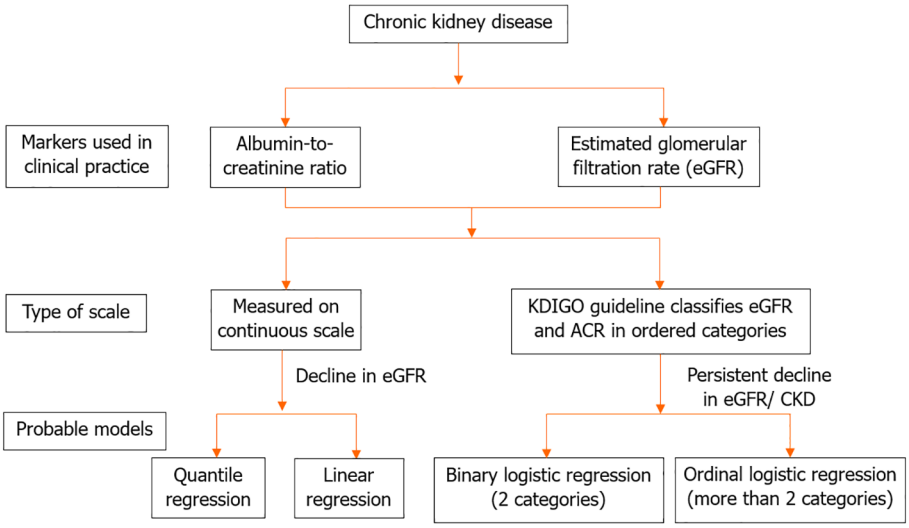

Figure 2 Selection of appropriate regression model for chronic kidney disease. ACR: albumin-to-creatinine ratio; KDIGO: Kidney Disease Improving Global Outcomes; eGFR: Estimated glomerular filtration rate.

relationship between the outcome and the predictor still holds in addition to various other assumptions of heteroskedasticity (differences in variance of errors), multicollinearity (correlation between independent variables (covariates, in case of multiple linear regression), and independence of observations[8]. The concept of simple linear regression can be extended to include multiple independent variables (multiple linear regression). However, the decline in kidney function is a multifactorial condition with the probability of being skewed[11]. For example, Zhang *et al*[12] reported the serum stem cell factor level as a predictor of decline in kidney function using multiple linear regression. They used a single-time assessment of eGFR, unlike what is recommended by KDIGO guidelines, to assess kidney health. Similarly, Cheung *et al* [13] identified risk factors of incident CKD by eGFR change, contrary to KDIGO recommendation. Another study[14] applied multiple linear regression to predict urine ACR in diabetes, which could not provide information on how much risk of kidney disease (categorized as persistent ACR ≥ 30 mg/g) was estimated in individuals with diabetes. These studies indicate the limitations of using multiple linear regression to predict CKD. The other strategy would be to overcome the stringent assumptions of linear regression; for this, the quantile regression method could be an alternative for CKD prediction, as discussed in the following paragraph.

Quantile regression model for CKD

The concept of quantile regression was given by Koenker and Bassett in 1978. The mathematical model for the quantile regression to estimate the qth quantile of the outcome variable Y and covariate X: $Q_{Y|X}(q) = f(\beta, X = x_i) = X\beta_q$, where, probability $(Y \leq f(\beta, X = x_i)) = q$ and β is regression coefficient, $0 \leq q \leq 1$.

Quantile regression models the quantile of the outcome variable and thus can handle skewed distribution of kidney function decline, with the assumptions of covariates being the same[8]. As for ordinary least square regression, quantile regression minimizes the weighted distances. Additionally, it is more robust and does not make any assumption about the distribution of the outcome variable, except the continuity of the variable, and can be used to model extreme values

[15,16]. However as discussed in linear regression, the issue of categorization of eGFR for KDIGO-based CKD classification cannot be neglected[12,14]. Nevertheless, it requires a larger sample size than linear regression[8].

REGRESSION MODELS BASED ON CATEGORICAL OUTCOME VARIABLES FOR CKD

Poisson regression model for CKD

Poisson regression was named after the French mathematician and physicist Siméon Denis Poisson. The Poisson regression model is given by: $Y_{i=}$ Log $(\lambda_i) = \beta_0 + \beta_i X_i$, where observed values Y_i~Poisson distribution with $\lambda = \lambda_i$, X_is are covariates, and β_is are regression coefficients.

Poisson regression is used to model the variable following the Poisson distribution under the assumption of equal mean and variance of the variable[17]. It was initially developed to model discrete outcome variables (count variable) but has also been widely accepted to model dichotomous variables (variables with binary outcome). Thus, Poisson regression could be an option to model the occurrence of CKD. However, since CKD has a low yearly incidence resulting in a maximum number of nondisease cases, the distribution of the outcome variable is skewed. This violates the assumption of equivalence of mean and variance. The incidence of CKD reported to date ranges from 0.49%/year to 1.9%/year in different disease groups[13,18-22]; *i.e.* approximately 1 in 100 individuals followed up for a year develops CKD and most of the participants remain disease free. This confirms the skewed distribution of the data with unequal mean and variance, limiting the use of Poisson regression for the predictive modeling of CKD. Various resources suggest the use of zero-inflated Poisson regression in case of overdispersion, as observed in the case of CKD[23]. However, zero-inflated models assume the presence of two processes behind the generation of added zeros; the unexplored area of CKD[24,25]. Thus, zero-inflated models could not apply to CKD. Negative binomial regression could be a more recommended technique for the predictive modeling of CKD; however, to model such cases whether the negative binomial regression model is better than the proportional odds model is still debatable[26].

Logistic regression model for CKD

The logistic regression model was primarily developed by Joseph Berkson where the relationship between the outcome variable Y and the covariate X is given by: $\text{Logit}\{Y|X\} = \text{logit}(P) = \log = X\beta$, where, $P = \text{Prob}\{Y = 1|X\}$ and β is the regression coefficient.

Logistic regression models the categorical outcome variable using the method of maximum likelihood estimation[8]. The three logistic regressions, binary, ordinal (proportional odds model), and multinomial, model three different types of outcome variables: dichotomous, ordinal and nominal, respectively. The sample size required for the diagnostic models needs to be such that the predictive model does not overfit the training data and is based on the event per predictor parameter and the number of predictors[27]. CKD is a multifactorial disease with poor awareness of its risk factors, especially in low-resource settings[28-30]. Thus, larger study cohorts with longer periods of follow-up are required to predict CKD, which is a challenge for low-resource settings. Although they have a few limitations, logistic regression models with penalized predictor effects can be used to partially overcome the issue of overfitting[31]. This agrees with the evidence from the existing literature[32]. In the case of small sample studies, internal validation using bootstrapping could be preferred for robust model estimates[33]. Table 1 shows the form of hypothetical data valid to be used for logistic regression.

CHALLENGES ASSOCIATED WITH PREDICTIVE MODELING

Overfitting in predictive models

As stated in the previous section, the regression model developed using a small sample size is usually overoptimistic and may not perform well in external validation (performance of the developed model in an independent cohort)[8]. Adequate sample size methods have been suggested to reduce overfitting[10,27,34]. Overfitting of the model also comes into play in cases of rare diseases with lower incidence where the potential risk factors of the disease could not be accurately estimated. The duration of diabetes plays a major role in the prediction of CKD[35]. However, with the poor awareness of diabetes, the correct reporting of the duration of diabetes is the major issue that may cause overfitting of the model due to an added potential predictor with suboptimal accuracy. Furthermore, chronic diseases like CKD are complexly affected by various demographic, biochemical, environmental, genetic and lifestyle-associated factors. Thus, chronic diseases with multiple confounding factors are prone to cause overfitting in their predictive models. To overcome this, several methods of penalization have been developed that shrink the coefficients of unimportant variables close to zero and thereby reduce the overfitting of the developed model. LASSO regression, elastic net, and Ridge regression are the available penalization methods that account for the overfitting of the model[8,36]. The global shrinkage factor of 0.9 is considered optimum, with bootstrapping considered the best method to calculate shrinkage post-estimation[27]. However, shrinkage methods have also been shown to fail in cases of small sample sizes[31]. Thus, using a lesser number of predictors, meaningful derivatives (variables calculated from several variables, like body mass index using height and weight) that combine several variables, and principal component analysis to reduce the number of covariates has been suggested[27]. Similar to the prediction of CKD, the modeling of time to the occurrence of CKD also suffers the limitation of overfitting. Thus, apart from dimension reduction techniques, penalization methods such as penalized maximum likelihood for binary logistic regression, and penalized likelihood in Cox regression were observed as a better-developed

Table 1 Hypothetical data format for the use of logistic regression model for chronic kidney disease

ID	Age (year)	Gender	eGFR1 (mL/min/1.73 m²)	eGFR_grade1[1]	eGFR2 (mL/min/1.73 m²)	eGFR_grade2[1]	Chronic kidney disease[2]
1	58	0	88.08	2	103.68	1	0
2	48	1	107.51	1	88.90	2	0
3	37	1	94.28	1	88.12	2	0
4	58	0	93.17	1	87.06	2	0
5	53	0	58.42	3	51.35	3	1
6	37	0	95.73	1	108.90	1	0
7	43	1	84.51	2	97.25	1	0
8	49	0	100.02	1	97.84	1	0
9	33	1	105.80	1	98.74	1	0
10	53	0	108.04	1	104.39	1	0
11	46	1	106.05	1	89.04	2	0
12	59	0	114.62	1	106.81	1	0
13	60	0	121.17	1	88.75	2	0
14	40	0	101.23	1	103.60	1	0
15	55	1	114.35	1	90.59	1	0
16	55	1	90.07	1	119.00	1	0
17	42	1	86.74	2	157.50	1	0
28	43	0	47.93	3	55.74	3	1
29	41	0	97.79	1	102.74	1	0
30	30	1	117.68	1	77.65	1	0

[1]> 2 ordered categories – ordinal logistic regression.

[2]2 categories – binary logistic regression.

1 coded for male and 0 coded for female. eGFR: Estimated glomerular filtration rate.

and more general shrinkage method[6,37].

Loss of information due to categorization of continuous variables

KDIGO classifies CKD using eGFR or ACR; defined as persistent eGFR < 60 mL/min/1.73 m² or ACR ≥ 30 mg/g for ≥ 3 mo[3]. The categorization of the variable measured on a continuous scale is done for the diagnosis of the disease or classification of the disease in different stages. Categorization is useful for descriptive purposes but may result in a loss of information for data analysis[8,38]. The comparison between studies can be efficiently made when the optimal cutoff point is available (as in the case of CKD); however, differences in the use of disease definitions restrict the generalization of the findings[39-41]. There is a lack of agreement between definitions of decline in renal function or CKD incidence[42, 43]. In most studies, the definition of CKD was taken as per the KDIGO guidelines (based on outcome), while in some, varying units of percentage decline in eGFR or increase in ACR were used to describe kidney disease. The decline of ≥ 15%, ≥ 30% and 40% to varying units of annual decline in eGFR is being used to define decrease in kidney function[39,44-47]. Similarly, the lack of agreement between studies also exists for a persistent decline in eGFR, or increase in ACR as many studies analyzed results with single-time assessment of ACR or eGFR. These differences in disease definitions and categorizations leads to comparisons between the studies being difficult, leading to information loss and biased conclusions.

For improved CKD prediction and categorization of the parameters to diagnose CKD, KDIGO guidelines need to be followed. Furthermore, it has been suggested that studies that define decline in renal function (in the case of CKD) by their median lead to the loss of power similar to loss incurred by a loss of a third of data from small studies[38,48]. Thus, dichotomization by median also leads to false-positive results, underestimation of the extent of variability in the variable, and misclassification of individuals with similar characteristics as being different[49]. However, studies suggest using three or more categories (preferably at percentiles), so that the apparent shape of the relationship between the variables under study can be inferred[38]. Nevertheless, the use of quartiles for the categorization of continuous variables remains debatable[50].

CONCLUSION

The prediction of disease is complex and requires several factors and rigorous methodology for the predictive model to be efficient, parsimonious and generalizable to a larger population of interest. To the best of our knowledge, this is the first review discussing the broad categories of predictive modeling methods and several other challenges associated with the prediction of CKD. The review discusses the various methodological challenges associated with several statistical models to predict CKD. Following KDIGO guidelines, eGFR or ACR needs to be categorized to define CKD in clinical and epidemiological settings. Thus, regression models, that could best study categorical outcome variables is the suggestive methodology for CKD modeling. Since the categorization of eGFR or ACR could not be neglected for the diagnosis of CKD, therefore linear regression or quantile regression cannot be used for the predictive modeling of CKD. Moreover, with the low early incidence of CKD, the assumption of equidispersion of Poisson regression cannot be achieved. Nevertheless, the clinical implication of these models could be achieved only if we adhere to the clinical practice guidelines formulated by KDIGO. Thus, in light of the review of existing literature, binary logistic regression seems to be the preferred method for the predictive modeling of CKD using the method of maximum likelihood estimation. Moreover, using appropriate shrinkage methods, penalized maximum likelihood estimates can be used to account for overfitting. Moreover, with the severity of CKD and its consequences on public health and challenges associated with its prediction, we anticipate that the predictive model of CKD could be accurate and specific with the inclusion of the important demographic, biochemical and molecular markers rather than being parsimonious to control the kidney disease burden.

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FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Quality of life and psychological distress in end-stage renal disease patients undergoing hemodialysis and transplantation

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Abstract

BACKGROUND

Among diverse profound impacts on patients' quality of life (QoL), end-stage renal disease (ESRD) frequently results in increased levels of depression, anxiety, and stress. Renal replacement therapies such as hemodialysis (HD) and transplantation (TX) are intended to enhance QoL, although their ability to alleviate psychological distress remains uncertain. This research posits the existence of a significant correlation between negative emotional states and QoL among ESRD patients, with varying effects observed in HD and TX patients.

AIM

To examine the relationship between QoL and negative emotional states (depression, anxiety, and stress) and predicted QoL in various end-stage renal replacement therapy patients with ESRD.

METHODS

This cross-sectional study included HD or TX patients in the Eastern Region of Saudi Arabia. The 36-item Short Form Survey and Depression Anxiety Stress Scale (DASS) was used for data collection, and correlation and regression analyses were performed.

RESULTS

The HD and TX transplantation groups showed statistically significant inverse relationships between QoL and DASS scores. HD patients with high anxiety levels and less education scored low on the physical component summary (PCS). In addition, the results of the mental component summary (MCS) were associated with reduced depression. Compared with older transplant patients, TX patients'

PCS scores were lower, and depression, stress, and negative working conditions were highly correlated with MCS scores.

CONCLUSION

The findings of this study revealed notable connections between well-being and mental turmoil experienced by individuals undergoing HD and TX. The PCS of HD patients is affected by heightened levels of anxiety and lower educational attainment, while the MCS of transplant patients is influenced by advancing age and elevated stress levels. These insights will contribute to a more comprehensive understanding of patient support.

Key Words: Anxiety; Depression; End-stage renal disease; Hemodialysis; Patient Reported Outcome Measures; Psychological distress; Quality of life; Renal replacement therapy outcomes; Saudi Arabia; Stress

Core Tip: This study examined the association between quality of life (QoL) and negative emotional states (depression, anxiety, and stress) in patients receiving end-stage renal replacement therapy (hemodialysis [HD] and transplantation [TX]) in Saudi Arabia. Using the 36-item Short Form Survey and Depression Anxiety Stress Scale, we discovered significant inverse correlations between QoL and emotional distress. High anxiety and lower educational levels have a negative impact on the physical component of QoL in HD patients, while older age and elevated stress levels affect the mental component in patients with TX.

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INTRODUCTION

There has been a recent increase in interest among patients undergoing rehabilitative interventions, such as kidney replacement therapy and transplantation, with the goal of improving their quality of life (QoL) and addressing associated mental stress. Recent data from the Global Burden of Disease Study reveals a growing prevalence of end-stage renal disease (ESRD). In 2019, approximately 17.3 million individuals worldwide were affected by chronic kidney disease (CKD) caused by glomerulonephritis, and projections suggest that this number will rise to 18.6 million by 2030[1]. Numerous studies have been conducted to assess the impact of these treatments on patients' psychological well-being and emotional health. Lyrakos *et al*[2] emphasized the significance of psychological support, noting that patients who received such support exhibited lower levels of anxiety, depression, and suicidal ideation. Similarly, Ratti *et al*[3] emphasized the role of social support in reducing psychological distress and depressive symptoms in ESRD patients.

Patients with ESRD experience substantial benefits from renal replacement therapy, which is considered a fundamental medical solution for improving their overall well-being. Nevertheless, the QoL for many individuals with advanced-stage or terminal kidney conditions is noticeably lower compared to the general population or those with other chronic disabilities. This decline in QoL can be attributed to several factors, such as inadequate fluid intake, dietary restrictions, and the daily adherence to prescribed medications[4].

Patients receiving renal replacement therapy, whether through hemodialysis (HD) or TX, often experience psychological distress due to factors such as physical pain, limited treatment options, and concerns about long-term health outcomes. Previous studies have shown that renal TX generally leads to a better QoL compared to HD[5]. However, a significant number of patients undergoing both treatments continue to report feelings of sadness and anxiety[6,7]. It has been recognized that providing psychological support is crucial in reducing anxiety levels among these patients. Both in-person and online counseling sessions have been shown to significantly decrease both trait and state anxiety levels[7,8]. Furthermore, psychosocial factors like somatization and mood disorders have been found to be associated with higher healthcare costs and poorer outcomes in kidney TX recipients. This highlights the importance of addressing the psychological well-being of this patient population[9].

Patients with ESRD have concerns about their overall physical and psychological well-being, as well as the particular effects of dialysis and TX. This research explores the link between ESRD patients' QoL and psychological health disorders (*i.e.* depression, anxiety, and stress [DAS]), using the Depression, Anxiety, and Stress Scale (DASS). The goal is to fill the knowledge gap regarding the intricate mechanisms that impact the health of ESRD patients receiving different renal replacement treatments.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Eastern Region of Saudi Arabia. The study participants consisted of patients diagnosed with ESRD undergoing renal treatment through either HD or TX. The data were collected using convenience sampling. This study was part of the "Quality of Life, Depression and Anxiety in Patients Undergoing Renal Replacement Therapies" research project[10]. A sample size calculation was conducted using a bivariate normal model with an exact distribution to examine a correlation coefficient of 0.2 in a one-tailed test, with an α error probability of 0.05 and a power of 0.80. The null hypothesis presumed no correlation (ρ = 0). The investigation concluded that 153 participants were necessary to attain adequate statistical power to meet the research objectives. The effect size for the correlation was computed and categorized following the guidelines outlined by[11]. A small effect size is represented by $R^2 = 0.01$, a medium effect size by $R^2 = 0.09$, and a large effect size by $R^2 = 0.25$.

The 36-item Short Form Survey is a widely used questionnaire established as a standard measure for various purposes. It is considered one of the most widely used assessments of health-related QoL and has a validated and reliable Arabic-language version[12]. This comprehensive measure evaluates eight dimensions pertaining to health: physical functionality, limitations caused by physical health issues, physical pain experienced, social capability, overall mental well-being, limitations arising from emotional problems, energy levels and fatigue (vitality), and the general perception of personal health[13].

The adapted Arabic version of the DASS was developed to measure the intensity of negative emotional states including DAS. This self-report questionnaire consists of 42 items and assesses the severity of depression, anxiety, and related symptoms over the past week. Respondents indicated the occurrence of symptoms, with each item rated from 0 (no stress or depression in the last week) to 3 (severe depression and stress in the last week)[14].

Ethical considerations

The study protocol was approved by the institutional review board at Imam Abdulrahman Bin Faisal University (Dammam, Saudi Arabia), subject to conventional expectations concerning voluntary participation and non-compromised care. All participants provided informed written consent, and data privacy was safeguarded through anonymization and secure storage methods. Data collection occurred during dialysis or clinic appointments, following the principles of the Declaration of Helsinki for Human Ethics and the Consent to Participate. Steps were taken to minimize participant discomfort and guarantee data confidentiality and security, while also addressing potential conflicts of interest or biases in accordance with ethical guidelines.

Statistical analyses

SPSS version 22 was used to conduct the descriptive analysis. Correlations were used to assess the association between the QoL scores and DAS. Regression analysis was used to predict the QoL scores in relation to DAS and demographic characteristics. Statistical significance was set at $P < 0.05$. Assumptions of normality, linearity, and homoscedasticity were assessed through Shapiro-Wilk tests, Q-Q plots, scatter plots of observed *vs* predicted values, and residual plots, guaranteeing the suitability of the employed statistical methods. The subscales pertaining to the QoL exhibited a normal distribution. In the TX group, mental component summary (MCS) scores displayed noteworthy linear correlations with DAS, while physical component summary (PCS) scores exhibited a significant linear relationship solely with anxiety. Regarding the HD group, MCS scores consistently exhibited significant linear associations with DAS, whereas PCS scores displayed significant relationships with depression and anxiety.

RESULTS

This study included 105 HD patients and 92 TX patients. Figure 1 summarizes key demographic data for both groups. There was a notably higher proportional of males among HD patients (76.2%) compared to TX patients (63.0%). Both groups had a fairly even distribution of married and unmarried participants. Patients with HD had higher rates of illiteracy (19.0% *vs* 4.3%) and lower rates of higher education (14.3% *vs* 26.1%) compared to TX patients. Rates for G1-8 and G9-12 education were similar between the groups, while diploma attainment rates were nearly identical. Non-employment rates were 58.1% for HD patients and 42.4% for TX patients. The majority of TX patients were of Saudi nationality (96.7%).

Table 1 shows the correlations between the QoL and DASS domains. In HD patients, there were noteworthy negative associations observed between scores reflecting QoL and indicators of DAS. Specifically, for the PCS, the correlations were found to be -0.404^1 ($R^2 = 0.163$), -0.314^1 ($R^2 = 0.099$), and -0.412^1 ($R^2 = 0.170$), respectively, denoting medium to large effect sizes. The MCS also exhibited notable correlations: -0.332^1 ($R^2 = 0.110$) for depression, -0.368^1 ($R^2 = 0.135$) for anxiety, and -0.240^2 ($R^2 = 0.058$) for stress. Conversely, in TX patients, significant associations were established for MCS: -0.544^1 ($R^2 = 0.295$) for depression, -0.388^1 ($R^2 = 0.151$) for anxiety, and -0.508 ($R^2 = 0.258$) for stress, all of which indicated large effect sizes. The correlations pertaining to PCS were comparatively smaller in magnitude, with only anxiety demonstrating significance at -0.248^2 ($R^2 = 0.062$).

By analyzing the QoL in different patient groups, the foremost influencers of QoL could be identified. In HD patients, increased anxiety and lower education levels have been found to negatively impact PCS and QoL. The comprehensive model showed statistical significance ($F = 6.07$, $P < 0.05$), and explained 47% of the variance in the PCS scores. In a broader patient cohort, depression was found to be a significant factor that negatively affected MCS. The overall model explained 25% of the variance in QoL ($R^2 = 0.25$, $F = 2.38$, $P < 0.05$). In TX patients, older age was associated with a lower

Table 1 Correlation between quality of life and depression anxiety stress scale domains

Domians	HD			TX		
	Depression (R^2)	Anxiety (R^2)	Stress (R^2)	Depression (R^2)	Anxiety (R^2)	Stress (R^2)
PCS	-0.404[1] (0.163)	-0.314[1] (0.099)	-0.412[1] (0.170)	-0.142 (0.020)	-0.248[2] (0.062)	-0.162 (0.026)
MCS	-0.332[1] (0.110)	-0.368[1] (0.135)	-0.240[2] (0.058)	-0.544[1] (0.295)	-0.388[1] (0.151)	-0.508[1] (0.258)

[1]Correlation is significant at the 0.01 level.
[2]Correlation is significant at the 0.05 level.
R^2 effect size. MCS: Mental component summary; PCS: Physical component summary.

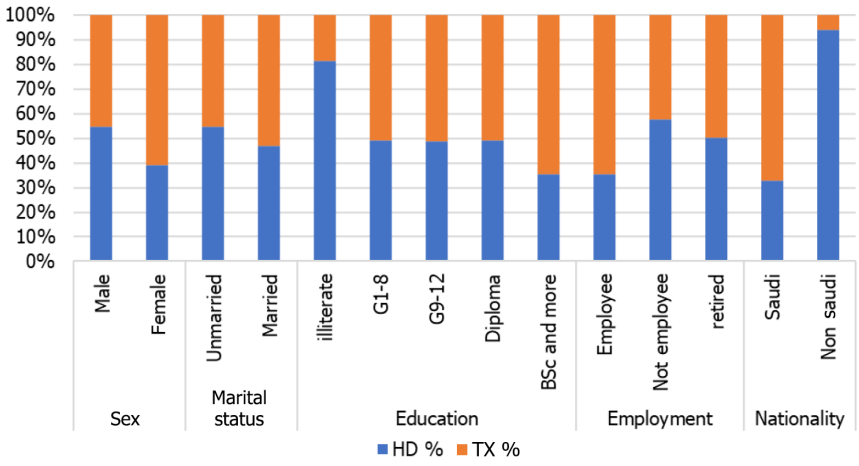

Figure 1 Demographic characteristics. HD: Hemodialysis; TX: Transplantation.

PCS score, with the model explaining 25% of the QoL variance (R^2 = 0.25, F = 2.24, P < 0.05). Ultimately, the mental wellbeing of TX recipients is adversely affected by heightened levels of depression, stress, and negative employment circumstances. MCS is specifically affected by psychological aspects of QoL. Through a comprehensive analysis, it was determined that the developed model could explain 66% of QoL outcomes' variance (Table 2).

DISCUSSION

This study investigated the complex relationship between psychological well-being and QoL in patients receiving end-stage renal replacement therapy for ESRD. Poor emotional status revealed a significant negative association between QoL and DAS in HD and TX patients. According to the regression analysis, greater anxiety and lower educational attainment were associated with lower PCS scores in HD patients, whereas depression influenced MCS scores. Older TX patients had lower PCS scores, with sadness, stress, and an unfavorable employment situation all having a substantial impact on MCS scores.

Comparative analysis of existing literature consistently highlights the detrimental impact of psychological distress on QoL in both HD and TX populations. Previous research consistently identifies high prevalence rates of anxiety (20%-45%) and depression (25%-50%) among HD patients, underscoring their vulnerability to mental health challenges[15-18]. Regression analysis further highlighted the negative association between higher anxiety[17,19-21].

The regression findings provide additional evidence of the distinct effects of anxiety, depression, and stress on PCS and MCS scores, underscoring the importance of implementing targeted interventions to enhance psychological well-being among patients undergoing renal replacement therapy[17,21-24]. Effectively addressing these mental health issues is essential for improving QoL and patient outcomes (and also healthcare system efficiency).

Moreover, the comparison between cohorts undergoing HD and TX highlights significant disparities in challenges and outcomes. TX, in contrast to dialysis, presents enhanced health status and potential employment advantages. However, it is crucial to prioritize ongoing care and management to maintain these improvements[25-27]. On the other hand, individuals undergoing HD often face enduring procedural difficulties, financial limitations, and social isolation, all of which contribute to inferior psychological well-being and diminished QoL[28]. Dialysis leads to social loneliness due to loss of independence, financial challenges, and limited daily activities. This persists even though TX improves health status and ongoing care is a priority[29].

Table 2 Multiple regression analysis predicting of quality of life among patients

Variable	HD						TX					
	PCS			MCS			PCS			MCS		
	B	*SE B*	*β*	*B*	*SE B*	*β*	*B*	*SE B*	*β*	*B*	*SE B*	*β*
Depression	-	-	-	-0.321	0.155	-0.309[a]	-	-	-	-0.516	0.238	-0.356[a]
Anxiety	-0.473	0.209	-0.302[a]	-	-	-	-	-	-	-	-	-
Stress	-	-	-	-	-	-	-	-	-	-0.465	0.183	-0.416[a]
Age	-	-	-	-	-	-	-0.180	0.080	-0.299[a]	-	-	-
Employment status	-	-	-	-	-	-	-	-	-	-3.077	1.282	-0.245[a]
Education level	-1.904	0.741	-0.220[a]	-	-	-	-	-	-	-	-	-
R2	0.47			0.25			0.25			0.66		
F	6.07			2.38			2.24			5.2		
Adj R2	0.39			0.15			0.14			0.36		

[a]$P < 0.05$.

HD: Hemodialysis; MCS: Mental component summary; PCS: Physical component summary; TX: Transplantation.

This study's limitations include its lack of a relatively large sample size, which inherently inhibits generalizing the outcomes. Furthermore, the cross-sectional design and reliance on self-reported measures may introduce various forms of bias. It is also important to consider the cultural and regional specificity of the studied context, which further limits the generalizability of the findings. Additionally, there may be unmeasured confounding variables, such as socioeconomic status and comorbidities, that could have an impact on pertinent variables, and thus the results. In future research, it would be beneficial to address these limitations and investigate the longitudinal effects of the variables in question.

CONCLUSION

These results provide important insights into the QoL of patients with ESRD. By examining the impact of dialysis on QoL, this study contributes to the knowledge base for healthcare managers and policymakers, enabling them to identify patient needs and vulnerable populations. The findings also suggest potential improvements that can be undertaken in hospital settings in order to enhance QoL. Additionally, by predicting QoL factors using psychological and demographic variables, this study increases awareness of relevant risk factors and assists administrators in implementing effective interventions. This research enhances our understanding of the QoL of ESRD patients, and emphasizes the need for further longitudinal studies, to comprehensively explore its complexity and changes over time.

FOOTNOTES

Author contributions: Shdaifat EA conceived and designed the study, conducted the research, provided research materials, and collected and organized the data; Shdaifat EA and Abu-Sneineh analyzed and interpreted the data; Shdaifat EA, Sudqi AM, and Abu-Sneineh FT wrote the initial and final drafts of the article; All authors critically reviewed and approved the final draft and were responsible for the content and similarity index of the manuscript.

Institutional review board statement: The study protocol priorities voluntary participant and non-compromised care, as approved by the Institutional Review Board (IRB) at Imam Abdulrahman Bin Faisal University (IRB-2017-04-089).

Informed consent statement: Informed consent was obtained from all participants and data privacy was ensured. Data collection took place while waiting for dialysis or clinic appointments, adhering to the principles of the Declaration of Helsinki for Human Ethics and the Consent to Participate. Measures were taken to minimize participant discomfort and ensure data confidentiality and security in accordance with the ethical guidelines.

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ORIGINAL ARTICLE

Prospective Study

Pilot study on the effect of flavonoids on arterial stiffness and oxidative stress in chronic kidney disease

Anastasia Vagopoulou, Panagiotis Theofilis, Despina Karasavvidou, Nasra Haddad, Dimitris Makridis, Stergios Tzimikas, Rigas Kalaitzidis

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Abstract

BACKGROUND

Flavonoids, the main class of polyphenols, exhibit antioxidant and antihypertensive properties.

AIM

To prospectively investigate the impact of flavonoids on arterial stiffness in patients with chronic kidney disease (CKD) stages I-IV.

METHODS

In this prospective, single-arm study, CKD patients with arterial hypertension and diabetes mellitus were enrolled. Baseline demographic, clinical, and laboratory variables were recorded. Patients received daily treatment with a phenol-rich dietary supplement for 3 months. Blood pressure, arterial stiffness (carotid-femoral pulse wave velocity, central pulse pressure), and oxidative stress markers (protein carbonyls, total phenolic compound, total antioxidant capacity) were measured at baseline and at study end.

RESULTS

Sixteen patients (mean age: 62.5 years, 87.5% male) completed the study. Following intervention, peripheral systolic blood pressure decreased significantly by 14 mmHg ($P < 0.001$). Carotid-femoral pulse wave velocity decreased from 8.9 m/s (baseline) to 8.2 m/s (study end) ($P < 0.001$), and central pulse pressure improved from 59 mmHg to 48 mmHg ($P = 0.003$). Flavonoids also reduced oxidative stress markers including protein carbonyls ($P < 0.001$), total phenolic compound ($P = 0.001$), and total antioxidant capacity ($P = 0.013$).

CONCLUSION
Flavonoid supplementation in CKD patients shows promise in improving blood pressure, arterial stiffness, and oxidative stress markers.

Key Words: Flavonoids; Chronic kidney disease; Arterial stiffness; Oxidative stress; Carotid-femoral pulse wave velocity

Core Tip: Daily flavonoid supplementation in patients with chronic kidney disease stage I-IV demonstrated significant reductions in peripheral systolic blood pressure, carotid-femoral pulse wave velocity, and central pulse pressure, along with improvements in oxidative stress markers. These findings suggest that flavonoids hold promise as an adjunctive therapy to manage hypertension, arterial stiffness, and oxidative stress in chronic kidney disease patients, potentially mitigating cardiovascular risk in this population.

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INTRODUCTION

Flavonoids, encompassing various subclasses such as flavonols, flavones, catechins, anthocyanins, isoflavones, dihydroflavonols, and chalcones, are prevalent in vegetables, fruits, nuts, spices, herbs, red wine, and tea[1]. As primary polyphenols, flavonoids exert pharmaceutical, antioxidant, anti-inflammatory, and cardioprotective effects[2]. Further studies support the diuretic action of the flavonoids *via* natriuresis as well as anti-diabetic effects[3]. Researchers have linked the diverse effects of flavonoids on vascular health, particularly arterial stiffness, an independent cardiovascular risk factor[4]. This study aimed to investigate the impact of flavonoids on arterial stiffness in patients with chronic kidney disease (CKD) stages I-IV following the intake of polyphenol-rich dietary supplements.

MATERIALS AND METHODS

Study design

This pilot prospective, single-arm intervention study took place at the Nephrology Clinic "C. Katsinas" of the General Hospital of Ptolemaida "Mpodosakeio" from November 2021 to April 2022. Patients meeting inclusion criteria [aged ≥ 18 years, cognitively competent, diagnosed with arterial hypertension (per European Society of Cardiology/European Society of Hypertension 2018 guidelines or on antihypertensive medication), type 2 diabetes mellitus, and CKD stage I-IV) participated. Exclusion criteria encompassed chronic atrial fibrillation, congestive heart failure New York Heart Association class III-IV, recent acute myocardial infarction or anginal symptoms, mental illness or dementia, active malignancy, alcohol abuse, inability/unwillingness to provide consent, or known flavonoid allergies.

Participants received a daily oral dietary supplement rich in phenols (200 mg) for 3 months. The supplement composition included fats (36.5 g/100 g), proteins (10 g/100 g), and carbohydrates (40.8 g/100 g), providing 532 kcal/100 g. Patients underwent clinical examinations, laboratory tests, and arterial stiffness assessments *via* carotid-femoral pulse wave velocity (cfPWV) at enrollment and after 3 months. Oxidative stress indicators (total phenols, total antioxidant capacity, protein carbonyls) were also evaluated.

All patients provided written informed consent after receiving comprehensive study information. The Ethics Committee of the General Hospital of Ptolemaida approved the study (80/18-11-2021), which adhered to the Declaration of Helsinki (1989).

Assessment of arterial stiffness

Arterial stiffness was evaluated by measurement of cfPWV at enrollment and 3 months later by an experienced operator blinded to the aims of the study. PWV measures the speed at which a pressure wave travels along the aorta and great arteries, reflecting the elasticity and compliance of these vessels. cfPWV is generally accepted as the "gold standard" measurement in evaluating aortic stiffness and is an important predictor of cardiovascular events. It can be calculated by dividing the distance traveled between two recording sites by the pulse transit time (PWV = distance in meters/by transit time in seconds). In this study, cfPWV was measured using the validated SphygmoCor device (AtCor Medical, IL, United States)[5-8]. All participants were positioned in a quiet environment with stable temperatures and rested in a supine position for a minimum of 10 min.

The measurements were conducted on the right common carotid and the right femoral artery. Participants were instructed to maintain silence during the measurements. Distances were measured directly with a tape measure: From the suprasternal notch to the femoral artery, and from the carotid artery to the suprasternal notch. The difference between these two measurements was calculated[6]. PWV was calculated by determining the pulse transit time and the distance between the two measurement sites. An electrocardiogram was recorded concurrently to align the pressure waves. All measurements were performed by the same experienced operator.

The radial pressure waveforms were calibrated using sphygmomanometric systolic and diastolic blood pressures taken from the brachial artery. The augmentation index of the central (aortic) pressure waveform was then estimated to assess wave reflection. This index reflects the interplay between wave reflection magnitude and arterial stiffness, with arterial stiffness influencing the timing of wave reflections.

Total phenols, total antioxidant capacity, and carbonyl

The concentration of total phenols was estimated according to the Folin-Ciocalteu method[9]. For the definition of the plasma total antioxidant capacity (TAC), the 2,2-diphenyl-1-picrylhydrazyl method was used. It is based on the use of the free radicals used for assessing the potential of substances to serve as hydrogen providers or free-radical scavengers. The results were shown as a percentage of antioxidant ability according to the following equation: AC% = (AT–AD) × 100/AT, where AT (means) represents the blind absorption to photometry to 520nm and AD represents sample absorption to photometry to 520 nm.

Finally, the concentration of carbonyls was detected based on their reaction with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone, and it was expressed as nmol/mL[10].

Statistical analysis

The normality of the distribution of continuous variables was assessed by the Shapiro-Wilk test. Continuous variables that follow a normal distribution were presented as mean ± SD. In the case of non-normally distributed continuous variables, data were presented as median with the interquartile range. Categorical variables were displayed as percentages. For the over-time comparison of normally continuous variables, we used the parametric paired sample *t*-test and the non-parametric Wilcoxon signed ranked test, accordingly. For the assessment of differences in cfPWV across the different CKD stages, a repeated measures analysis of variance was conducted, using the Benferroni correction.

All statistical calculations were performed using SPSS software (version 25.0; SPSS Inc., Chicago, IL, United States). All reported *P* values were based on two-sided hypotheses, with a *P* value of 0.05 being considered statistically significant.

RESULTS

Seventeen patients participated in the study between November 2021 and April 2022. One patient died due to acute myocardial infarction, and 16 patients completed the study. The mean age of the participants was 62.5 years and 87.5% were male. The remaining baseline characteristics and laboratory tests of the study population are presented in Tables 1 and 2. During the study, we did not observe any significant changes in serum creatinine of the participants ($P = 0.75$).

The effect of flavonoids on arterial stiffness measurements

The alterations in arterial stiffness measurements following the administration of flavonoids are presented in Table 3. After the intervention, there was a statistically significant reduction in the peripheral systolic blood pressure by 14 mmHg ($P < 0.001$). The mean cfPWV at baseline was estimated at 8.9 m/s (6.7, 11.8) and was markedly reduced to 8.2 m/s (5.1, 9.2); a difference that was statistically significant ($P < 0.001$) (Figure 1). The central pulse pressure was reduced after flavonoid administration from 59 mmHg (44, 69) at baseline to 48 mmHg (37, 60) at the end of the study ($P = 0.003$).

We also examined whether the alteration of cfPWV differed proportionally according to the stage of CKD. The analysis showed that the changes in cfPWV differed significantly among the CKD stages ($P = 0.037$) (Figure 2). After pairwise analysis among the CKD stages and the alteration of cfPWV, a statistically significant difference was noted between stages I and IIIb-IV ($P = 0.042$).

The effect of flavonoids on oxidative stress parameters

The changes in parameters of oxidative stress before and after flavonoid administration are displayed in Table 4. After the flavonoid administration, a reduction of plasma proteinic carbonyl was observed (73.50 ± 18.65 nmol/mL at baseline *vs* 52.54 ± 25.04 nmol/mL at the 3-month follow-up, $P < 0.001$). Moreover, the total phenolic compound concentration was significantly enhanced [25.11 mg/mL (16.95, 30.29) at baseline *vs* 31.91 mg/mL (30.49, 47.51) at the 3-month follow-up, $P = 0.001$]. The TAC also appeared augmented after the intervention [3.55% (1.15, 6.38) at baseline *vs* 12.51% (6.26, 17.66) at the 3-month follow-up, $P = 0.013$].

DISCUSSION

According to the recent guidelines for the non-pharmaceutical interventions of the International Society of Hypertension [11], antioxidant therapeutics are recommended as a means to reduce the production of reactive oxygen species and oxidative stress. It is well known that the abundance of reactive oxygen species and the promotion of oxidative stress play

Table 1 Baseline characteristics of the study population

Parameter	Value
Age, years	62.5 ± 8.2
Male	14 (87.5)
CKD stage	
I	6 (37.5)
II-IIIa	6 (37.5)
IIIb-IV	4 (25.0)
$eGFR_{CKD-EPI}$, mL/min/1.73m^2	84 (47.50, 98.75)

Data are *n* (%). CKD: Chronic kidney disease, eGFR: Estimated glomerular filtration rate.

Table 2 Baseline laboratory tests of the study population

Parameter	Value
Hct, %	42.3 ± 5.2
Hb, g/dL	14.3 ± 1.9
PLT, 10^3/μL	222 ± 51
Urea, mg/dL	51 (26, 70)
Creatinine, mg/dL	1.3 (0.77, 1.52)
Urine albumin, mg/24 h	183 (91.2, 949.0)
Sodium, mmol/L	139 ± 2
Potassium, mmol/L	4.9 (4.72, 5.00)
LDH, IU/L	183 ± 43
AST, IU/L	23 (15, 27)
ALT, IU/L	30 (18, 37)
Total serum protein, g/dL	6.7 ± 0.7
Serum albumin, g/dL	3.9 ± 0.8
Total cholesterol, mg/dL	152 ± 41
LDL-C, mg/dL	80 ± 31
Triglycerides, mg/dL	148 ± 59
Uric acid, mg/dL	5.8 ± 1.6
Calcium, mg/dL	9.6 (9.4, 9.9)
PTH, pg/mL	68 (40, 72)
CRP, mg/dL	0.52 (0.06, 0.31)
Fe, mg/dL	86 ± 32
Ferritin, ng/mL	201 (97, 299)
TIBC, mg/dL	288 (241, 341)
HbA1c, %	7.58 ± 1.63

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CRP: C reactive protein; Hb: Hemoglobin; HbA1c: Glycated hemoglobin; Hct: Hematocrit; LDH: Lactate dehydrogenase; LDL-C: Low-density lipoprotein-cholesterol; PLT: Platelet; PTH: Parathyroid hormone; TIBC: Total iron binding capacity.

Table 3 Changes in parameters of arterial stiffness at baseline and after treatment with flavonoids

Parameter	Baseline	End of study	*P* value
PSBP, mmHg	148 ± 16	134 ± 13	< 0.001
PDBP, mmHg	81.00 ± 8.00	76.00 ± 6.32	0.051
cfPWV, m/s	8.85 (6.7, 11.8)	8.20 (5.1, 9.2)	< 0.001
CSBP, mmHg	156 ± 23	137 ± 16	0.004
CDBP, mmHg	90 ± 14	80 ± 10	0.002
CPP, mmHg	59 (44, 69)	48 (37, 60)	0.003
CHR, pulse/min	69 ± 14	66 ± 9	0.370
AIx, %	26.5 ± 10.0	32.5 ± 13.3	0.510
Ap	17.44 (9.50, 21.00)	14.25 (9.50, 18.75)	0.780

AIx: Augmentation index; Ap: Augmentation pressure; CDBP: Central diastolic blood pressure; cfPWV: Carotid-femoral pulse wave velocity; CHR: Central heart rate; CPP: Central pulse pressure; CSBP: Central systolic blood pressure; PDBP: Peripheral diastolic blood pressure; PSBP: Peripheral systolic blood pressure.

Table 4 Changes in parameters of oxidative stress at baseline and after treatment with flavonoids

Parameter	Baseline	End of study	*P* value
Proteinic carbonyls, nmol/mL	73.50 ± 18.65	52.54 ± 25.04	< 0.001
TPC, mg/mL	25.11 (16.95, 30.29)	31.91 (30.49, 47.51)	0.001
TAC, %	3.55 (1.15, 6.38)	12.51 (6.26, 17.66)	0.013

TAC: Total antioxidant capacity; TPC: Total phenolic compound.

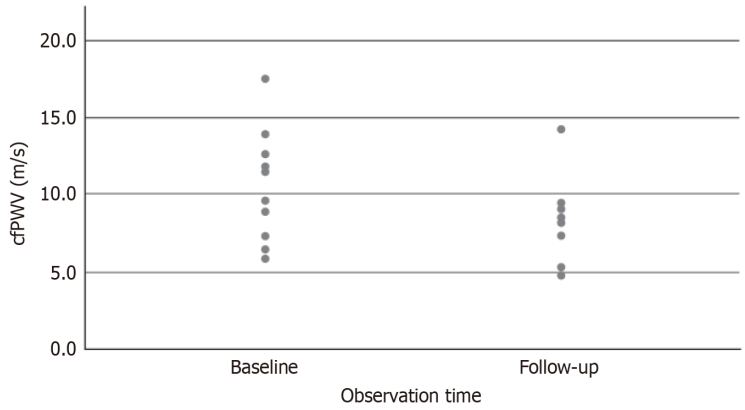

Figure 1 Scatter plot demonstrating the significant differences in carotid-femoral pulse wave velocity at baseline (first visit) and at the end of the study (3 months later). cfPWV: Carotid-femoral pulse wave velocity.

an important role in the pathogenesis of hypertension[12]. This phenomenon further leads to endothelial dysfunction and an impaired balance of vasoactive compounds such as the vasodilating nitric oxide and the vasoconstrictive angiotensin II and endothelin[12,13].

Polyphenols, which are commonly known antioxidant substances, have proven their beneficial role in hypertension [14]. The antihypertensive effect of flavonoids is mostly based on the protection of the endothelium[15] and the inhibition of the renin-angiotensin-aldosterone system[16]. In our study, we noted significant alterations in the parameters of diastolic blood pressure and central pressure after 3 months of treatment with flavonoids. Such findings are in line with previously published data[17].

Critically, we also observed a significant amelioration of arterial stiffness measures after treatment with flavonoids. Several studies have reported that flavonoids may affect arteriosclerosis and arterial stiffness. Specifically, Curtis *et al*[18] noted a reduction of PWV and central pressure in post-menopausal females with the administration of chocolate rich in

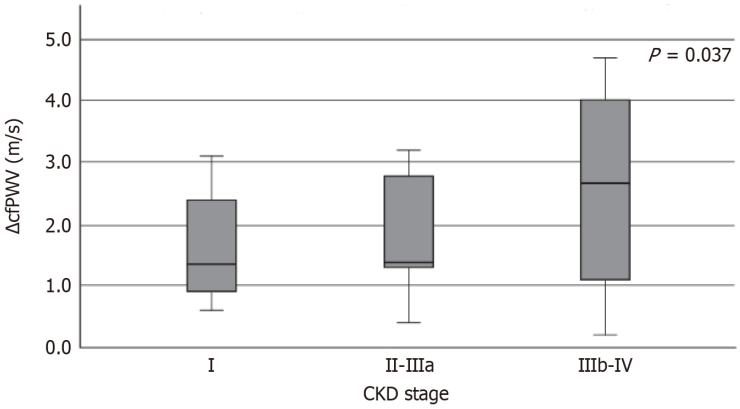

Figure 2 Boxplots of the alteration in carotid-femoral pulse wave velocity according to chronic kidney disease stage. cfPWV: Carotid-femoral pulse wave velocity; CKD: Chronic kidney disease.

cocoa with flavonoids after a 1-year follow-up. Nestel *et al*[19] made similar conclusions since they observed a reduction in PWV and blood pressure after the consumption of flavonoids by overweight males and post-menopausal females. The same reduction in PWV after the intake of flavonoids was observed in the general population. However, evidence is lacking in the CKD population. Our study is the sole prospective study to assess the importance of flavonoid administration in patients with CKD.

It is noteworthy that different types of flavonoids have been used as antioxidants across the studies. In this study, we utilized a flavonoid mixture containing cocoa, lemon balm rich in rosmarinic acid, caffeic acid, rockrose, and pomegranate extract. The caffeic acid has been studied for its antioxidant effects, which stem from the improvement in nitric oxide bioavailability, resulting in the prevention of cardiovascular and kidney diseases derived from oxidant stress[20]. Rockrose contains kaempferol and quercetin. Kaempferol, owing to its anti-inflammatory and antioxidant actions, reduces the accumulation of collagen in vessels and angiotensin II-induced inflammation and oxidative stress in heart failure[21]. Quercetin has strong antioxidant effects, mostly acting in brain vessels[22].

Flavonoids act as exogenous antioxidants due to their ability to give electrons to the roots of hydrogen Peroxide, hydroxyl, and hyperoxyl, stabilizing the aforementioned roots by reducing the levels of free radicals in the human body [23]. In our study, after the administration of flavonoids, we observed a reduction of the plasma proteinic carbonyls and an increase of the total phenolic content and TAC. Our data suggests that the antioxidant effects of flavonoids may also extend to patients with CKD. Previous studies have assessed the effects of polyphenols and especially flavonoids in CKD [17]. Cao *et al*[17], in a recent review, analyzed the effects of flavonoids in different types of CKD such as diabetic nephropathy, glomerulonephritis, and lupus nephritis. There seems to be an improved antioxidant effect in patients with CKD, which appears to be sufficient in decelerating the progression of the disease. In our study, this hypothesis could not be adequately tested due to the small sample size and the brief period of observation (3 months).

CONCLUSION

In conclusion, this pilot study showed that the administration of flavonoids in patients with CKD appears to have a positive effect on blood pressure, arterial stiffness, and oxidative stress measures. Future studies are needed to further test their potential in this patient population.

FOOTNOTES

Author contributions: Karasavvidou D conceived the study; Kalaitzidis RG supervised the study; Vagopoulou A, Karasavvidou D, Haddad N, Makridis D, and Tzimikas S contributed to the investigation; Vagopoulou A contributed to data curation and formal analysis; Vagopoulou A, Karasavvidou D, Haddad N, Makridis D, and Tzimikas S wrote the original draft; Theofilis P contributed to the visualization; Theofilis P and Kalaitzidis RG reviewed and edited the original draft; All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Protective effect of long-chain polyunsaturated fatty acids on hepatorenal syndrome in rats

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Abstract

BACKGROUND

Hepatorenal syndrome (HRS) is the most prevalent form of acute kidney injury in cirrhotic patients. It is characterized by reduced renal blood flow and represents the most severe complication in cirrhotic patients with advanced disease. Previous research has indicated that antioxidants can delay the onset of a hyperdynamic circulatory state in cirrhosis and improve renal function in HRS patients. Regular omega-3 supplementation has significantly reduced the risk of liver disease. This supplementation could represent an additional therapy for individuals with HRS.

AIM

To evaluated the antioxidant effect of omega-3 polyunsaturated fatty acid supplementation on the kidneys of cirrhotic rats.

METHODS

Secondary biliary cirrhosis was induced in rats by biliary duct ligation (BDL) for 28 d. We used 24 male Wistar rats divided into the following groups: I (control); II (treated with omega-3, 1 g/kg of body weight); III (BDL treated with omega-3, 1 g/kg of body weight); and IV (BDL without treatment). The animals were killed by overdose of anesthetic; the kidneys were dissected, removed, frozen in liquid nitrogen, and stored in a freezer at -80°C for later analysis. We evaluated oxidative stress, nitric oxide (NO) metabolites, DNA damage by the comet assay, cell viability test, and apoptosis in the kidneys. Data were analyzed by one-way analysis of variance, and means were compared using the Tukey test, with $P \leq$

0.05.

RESULTS

Omega-3 significantly decreased the production of reactive oxygen species ($P < 0.001$) and lipoperoxidation in the kidneys of cirrhotic rats treated with omega-3 ($P < 0.001$). The activity of the antioxidant enzymes superoxide dismutase and catalase increased in the BDL+omega-3 group compared to the BDL group ($P < 0.01$). NO production, DNA damage, and caspase-9 cleavage decreased significantly in the omega-3-treated BDL group. There was an increase in mitochondrial electrochemical potential ($P < 0.001$) in BDL treated with omega-3 compared to BDL. No changes in the cell survival index in HRS with omega-3 compared to the control group ($P > 0.05$) were observed.

CONCLUSION

The study demonstrates that omega-3 can protect cellular integrity and function by increasing antioxidant enzymes, inhibiting the formation of free radicals, and reducing apoptosis.

Key Words: Long-chain polyunsaturated fatty acids; Antioxidant effect; Hepatorenal syndrome; Liver cirrhosis; Reactive oxygen species; Apoptosis

Core tip: Hepatorenal syndrome (HRS) is associated with poor prognosis in individuals with advanced or decompensated cirrhosis. Palliative care involves the use of vasoconstrictor agents and intravenous albumin in combination. Currently, there are no effective treatments for this condition other than liver transplantation. Our research has shown that administering 1 g/kg of omega-3 to cirrhotic rats reduced oxidative damage, DNA damage, and apoptosis while enhancing antioxidant defenses and maintaining the kidney's cellular integrity. These findings indicate that omega-3 supplementation could be complementary to managing HRS.

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INTRODUCTION

Cirrhosis is a complex disease characterized by necroinflammation and fibrogenesis of the liver caused by different mechanisms, such as drug-induced hepatotoxicity, hepatitis B or C infections, excessive alcohol consumption, primary metabolic diseases, obesity, nonalcoholic fatty liver disease (NAFLD), autoimmune diseases, cholestatic diseases, and iron or copper overload[1-3].

In the course of the disease, hepatocytes and collagen deposition are continuously destroyed, replacing healthy liver parenchyma with fibrotic tissue and regenerative nodules. These events cause a pronounced distortion of the hepatic vascular architecture, which results in increased resistance to portal blood flow and, therefore, portal hypertension (PH), in addition to loss of liver function[3,4].

One of the main consequences in the advanced stage of cirrhosis is the development of hepatorenal syndrome (HRS), one of the most common types of kidney damage that affects patients with severe and decompensated liver disease and is characterized by decreased renal perfusion and vasoconstriction[5-7].

Few studies have analyzed the effect of treatment with antioxidant substances on the kidneys of cirrhotic animals since there is a consensus that renal dysfunction in cirrhosis results from hemodynamic changes and the activation of compensatory hormonal mechanisms. Previous studies have already demonstrated that, although there are no detectable structural changes in the kidney tissue of cirrhotic animals that underwent biliary duct ligation (BDL), there is a loss of integrity and cellular function in the kidney, with apoptosis, activation of caspase-3, oxidative damage to cell membranes and DNA damage[8].

BDL is a well-established model for cirrhosis development in 4 wk after surgery. The cirrhosis observed in animals shows damage in the liver parenchyma, the presence of fibrosis, PH, and ascites[9,10]. These conditions, mainly the presence of PH, are what lead to renal changes, with systemic hypotension, reduced glomerular filtration, and activation of the renin–angiotensin–aldosterone system. These data are widely recorded in current scientific literature, allowing the BDL model to study the HRS commonly seen in cirrhotic patients[11-15].

Omega-3 polyunsaturated fatty acids modulate the immune response by altering membrane phospholipids and preventing a proinflammatory state[16]. The benefit of omega-3 supplementation is well established in cardiovascular risk protection, mainly due to the reduction in triglycerides and low-density lipoproteins associated with omega-3 intake [17,18]. Other benefits regarding regular omega-3 intake are also reported, including a reduction in reactive oxygen

species (ROS) and C-reactive protein, as well as the decreased release of cytokines and other inflammatory mediators[16,19]. Another factor directly related to HRS is PH, characterized by an excessive increase in portal venous pressure and the development of portosystemic collaterals, diverting portal blood flow to the systemic circulation. Several factors can trigger PH; however, liver cirrhosis is the leading cause, comprising > 90% of cases of PH. Among the main complications resulting from PH is upper gastrointestinal bleeding due to the rupture of gastroesophageal varices. Another less critical complication is the presence of ascites[20,21].

In liver diseases, some studies have reported a beneficial action of omega-3 polyunsaturated fatty acids, reducing sepsis and inflammatory markers in patients with severe acute hepatitis[22], reducing inflammation and fibrosis in patients with biliary atresia[23], and decreasing the percentage of fat liver in patients with nonalcoholic steatohepatitis treated with purified docosahexaenoic acid (DHA)[24]. A significant reduction in the risk of liver disease, particularly NAFLD, has been demonstrated with regular omega-3 supplementation[19].

In this sense, regular use of omega-3 is closely related to preventing and reducing the severity of several diseases. Omega-3 fatty acids are found in the largest amounts in walnuts, flaxseed, chia, sardines, salmon, and tuna[25]. Omega-3 supplementation may represent an additional therapy for individuals affected by HRS since the effectiveness of vasoconstrictor and albumin treatment is limited to less than half of patients with HRS-acute kidney injury (AKI)[7], and liver transplantation may not be a reality for all patients. However, little is known about the role of these fatty acids in patients with HRS, including the effects on the concentrations of inflammatory cytokines and the improvement in the disease's course.

This study aimed to investigate the effects of supplementation with omega-3 fatty acids on oxidative stress and apoptosis in the kidney tissue of cirrhotic animals.

MATERIALS AND METHODS

Animals

All experimental procedures carried out on animals are by the recommendations of the Arouca Law (Law n° 11794, of 10/08/2008) and were approved by the Federal University of Health Sciences of Porto Alegre (UFCSPA) Ethics Committee with number 146/12. The animals were acclimatized to laboratory conditions (22 ± 2 °C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk before experimentation. BDL was performed under anesthesia, and the animals received analgesia for 48 h after surgery. Intragastric gavage administration was carried out with conscious animals, using straight gavage needles appropriate for the animal size. All animals were killed by an overdose of anesthetics.

In vivo studies

Twenty-four adults male Wistar rats with an average weight of 300 g were used. Liver cirrhosis was induced by the BDL model. Rats were anesthetized with 2% xylazine hydrochloride (50 mg/kg) and ketamine hydrochloride (100 mg/kg) (i.p.). Secondary biliary cirrhosis was induced in animals by double ligation and a total section of the common bile duct. The animals were killed 28 d after obstruction; a time in which there is complete development of both cholestasis and fibrosis and clear establishment of the biochemical changes characteristic of liver cirrhosis[26]. Animals were divided into four groups (n = 6): I (control treated with saline); II (treated with omega-3, 1 g/kg/d); III (BDL treated with omega-3, 1 g/kg/d); and IV (BDL treated with saline) (Figure 1). Daily omega-3 supplementation began on day 14 after BDL and continued for 2 wk until the death of the animals on day 28 after BDL.

Histological analysis

Five-micrometer sections of formalin-fixed and paraffin-embedded kidney slices were routinely processed with hematoxylin–eosin (performed by the Laboratory of Pathology at UFCSPA). A single pathologist, blinded to the experimental protocol, analyzed all kidney fragments using light microscopy.

Tissue and homogenate preparation

Animals were killed by an overdose of anesthetic. The frozen kidneys from each rat were homogenized in an ice-cold phosphate buffer and diluted in 10 volumes (1:10 w/v) of 20 mmol/L sodium phosphate buffer, pH 7.4, containing 140 mmol/L KCl. Homogenates were centrifuged at 750 × g for 10 min at 4 °C, the pellet was discarded, and the supernatant was immediately separated and used for the measurements.

Free radical levels

Homogenates were overlayed with 100 µL 25 µM dichlorofluorescein diacetate (DCFDA) and placed back in the incubator for 30 min at 37 °C. At the end of the incubation period, plates were removed, and the fluorescence of the homogenates was measured on a SpectraMax M2e Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). The excitation/emission wavelengths for DCFDA were 480/520 nm. Relative fluorescence (RFU) values were expressed as RFU/mg protein[27].

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS), a measure of lipid peroxidation, were determined according to Esterbauer and Cheeseman[28]. Homogenates were mixed with 10% trichloroacetic acid and 0.67% TBARS and heated in

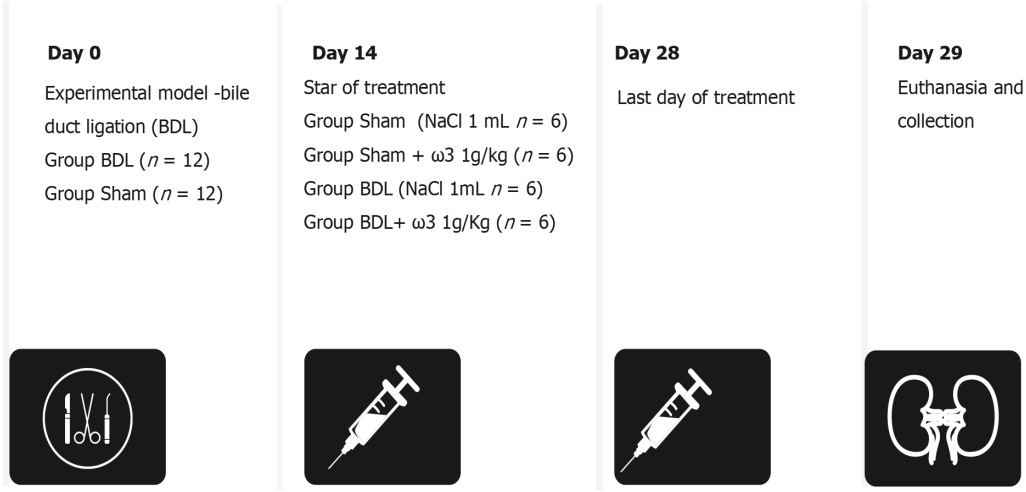

Figure 1 Experimental protocol of omega-3 administration.

a water bath for 25 min. The absorbance determined TBARS at 535 nm. Results were reported as nmol TBARS/mg protein.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity was evaluated by quantifying the inhibition of superoxide-dependent autoxidation of epinephrine and verifying the absorbance of the samples at 480 nm[29]. Briefly, 20 µL homogenate was added to 170 µL of a mixture containing 50 mmol/L glycine buffer pH 10.2 and 10 mmol/L catalase (CAT). After that, 10 µL epinephrine was added, and the absorbance was immediately recorded every 30 s for 12 min at 480 nm in a SpectraMax M2e Microplate Reader (Molecular Devices). The inhibition of autoxidation of epinephrine occurs in the presence of SOD, whose activity can then be indirectly assayed spectrophotometrically. One SOD unit was defined as the amount of SOD necessary to inhibit 50% of epinephrine autoxidation, and the specific activity is reported as SOD units/mg protein.

CAT

CAT activity was assayed according to the method described by Chance and Machley[30], based on the disappearance of at 240 nm. Briefly, 10 µL homogenate was added to 180 µL 20 mmol/L potassium phosphate buffer, pH 7.2. Subsequently, 10 µL 5 mmol/L H_2O_2 was added, and the absorbance was immediately recorded every 30 s for 10 min using a SpectraMax M2e Microplate Reader (Molecular Devices. One CAT unit was defined as 1 µmol H_2O_2 consumed per minute, and the specific activity is calculated as CAT units/mg protein.

Measurement of nitric oxide production

The production of nitric oxide (NO) was estimated by measuring the amount of nitrite, a stable metabolite of NO[31]. Briefly, 100 µL homogenate was mixed with 100 µL Griess reagent (1% sulfanilamide; 0.1% naphthyl ethylenediamine; 2.5% H_3PO_4) at ambient temperature. After 20 min, absorbance was measured at 540 nm using a SpectraMax M2e Microplate Reader (Molecular Devices). A nitrite calibration curve was used to convert absorbance to µM nitrite.

Alkaline comet assay

The alkaline comet assay was performed as previously described by Singh *et al*[32]. Briefly, 10 µL cells were mixed with 90 µL LMP agarose, spread on a standard agarose precoated microscope slide, and placed at 4 °C for 5 min to allow for solidification. Cells were lysed in high salt and detergent concentrations (2.5 M NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris with 1% Triton X-100 and 10% DMSO freshly added) for 2 h. Slides were removed from the lysing solution and washed three times with PBS. Subsequently, cells were exposed to alkali conditions (300 mmol/L NaOH/1 mmol/L Na_2 EDTA, pH > 13, 30 min, 4 °C) to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 25 min at 25 V and 300 mA (94 V/cm). After electrophoresis, the slides were neutralized and silver-stained [33]. One hundred cells were scored visually according to the tail length and the amount of DNA in the tail. Each comet was given an arbitrary value of 0–4 (0, undamaged; 4, maximally damaged), as described by Collins *et al*[34]. Damage score was thus assigned to each sample and ranged from 0 (completely undamaged: 100 cells 0) to 400 (with maximum damage: 100 cells 4). International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it highly correlates with computer-based image analysis[33,34].

Cell viability assay

Cell viability was determined by a trypan blue dye-exclusion assay (TBDE) used as a cytotoxic measurement[8]. Trypan blue staining is a long-standing and widely used method to identify dead cells. Only cells with intact membranes can effectively exclude the dye, so dead cells with compromised membranes become stained. Homogenates were mixed with 0.4% trypan blue solution for each group, which was then added. Cytotoxicity (cellular growth inhibitory rate) was

determined from the number of viable cells (no color) in treated samples as a percentage of the PBS control. We used the Countess® Automated Cell Counter (Invitrogen, Carlsbad, CA, USA).

Assessment of apoptosis by flow cytometric analysis

Annexin V-PE was used with a vital dye, 7-AAD, to distinguish apoptotic (Annexin V-PE positive, 7-AAD negative) from necrotic (Annexin V-PE positive, 7-AAD positive) cells. After treatment, cells were collected and resuspended in 40 µL binding buffer with 2 µL Annexin V-PE. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µL binding buffer and 2 µL 7-AAD were added. Cells were incubated for 5 min, and an additional 200 µL binding buffer was added. Before analysis, cells were filtered through a cell strainer cap fitted to a polystyrene round bottom flow cytometric tube. Data were collected and analyzed by a FACS Calibur flow cytometer with CellQuest software in a total of 10 000 events per sample; fluorescence was measured, and the percentage of viable, early apoptotic, late apoptotic, and necrotic cells was determined[35].

Quantification of cleaved caspase 9 by flow cytometric analysis

After treatment, cells were harvested and resuspended in 25 µL PBS and fixed with 4% formaldehyde. After permeabilization and blocking (0.2% Triton X-100 in PBS and 1% BSA), cells were incubated with the primary antibodies diluted 1:1000 for 1 h at room temperature, followed by incubation with anti-rabbit FITC secondary antibody at 1:1000 for 1 h at room temperature in the dark. Fluorescence intensity in arbitrary units was plotted in histograms, and the mean fluorescence intensity was calculated using CellQuest software.

Assay of electrochemical potential

After treatment, cells were incubated with 2 mmol/L Rh123 (rhodamine 123) for 30 min at 37 °C, washed, and resuspended in 100 µL PBS. The mitochondrial electrochemical potential was correlated to the fluorescence intensity of Rho123 (with decreased fluorescence signifying loss of the mitochondrial electrochemical potential)[36]. Flow cytometry was performed using a FACS Calibur (Becton Dickinson, San Jose, CA, USA) with excitation at 488 nm and emission read using a 525–550 nm filter (FL1).

Protein determination

Protein was measured using the method of Lowry *et al*[37], using bovine serum albumin as a standard.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), and means were compared using the Tukey test, with $P \leq 0.05$ considered statistically significant, using GraphPad Prism version 5.0 Program (Intuitive Software for Science, San Diego, CA, United States). All data were expressed as mean ± SEM. All analyses were performed in duplicate. The statistical methods of this study were reviewed by the statistical office of the Federal University of Health Sciences of Porto Alegre-NUPESQ-UFCSPA (https://ufcspa.edu.br/pesquisa-e-inovacao/apoio-a-pesquisa/assessoria-estatistica).

RESULTS

Histological evaluation of the liver of animals with BDL showed a loss of typical architecture and the presence of regenerative nodules, cellular necrosis, and fibrosis (data not shown). No alterations in renal histology were observed in bile duct ligated rats compared to sham-operated animals, as shown in Figure 2. The exclusion of structural kidney damage is a crucial component in HRS diagnosis. We did not perform other markers of renal function assessment, as the BDL model is widely accepted and validated for the experimental study of HRS.

Initially, we investigated the effect of exposure to omega-3 on some parameters of oxidative stress in rat kidneys. As shown in Figure 3, omega-3 decreased ROS production significantly ($P < 0.001$), and TBARS level was reduced by omega-3 ($P < 0.001$) in HRS.

We also observed an increase in mitochondrial electrochemical potential by omega-3 ($P < 0.01$) in HRS (Figure 4).

Regarding antioxidant enzymes (Figure 5), omega-3 increased SOD activity ($P < 0.01$). Control plus omega-3 did not alter activity of this enzyme ($P > 0.05$). CAT activity was significantly increased by omega-3 ($P < 0.001$). Figure 6 shows omega-3 inhibited NO production ($P < 0.05$).

To assess the DNA damage, we performed an alkaline comet assay. Figure 7A shows that omega-3 significantly decreased DNA damage ($P < 0.001$). No changes in HRS omega-3 were observed compared to the control group ($P > 0.05$).

Omega-3 treatment preserved cellular integrity in the kidneys of cirrhotic animals, as observed by cell viability determined by a TBDE (Figure 7B).

To delineate the mechanism of omega-3 after treatment, we monitored the caspase-9 activity (Figure 7C). Caspase-3 did not change at 1 h ($P > 0.05$). Caspase-9 was decreased by omega-3 in HRS ($P < 0.001$).

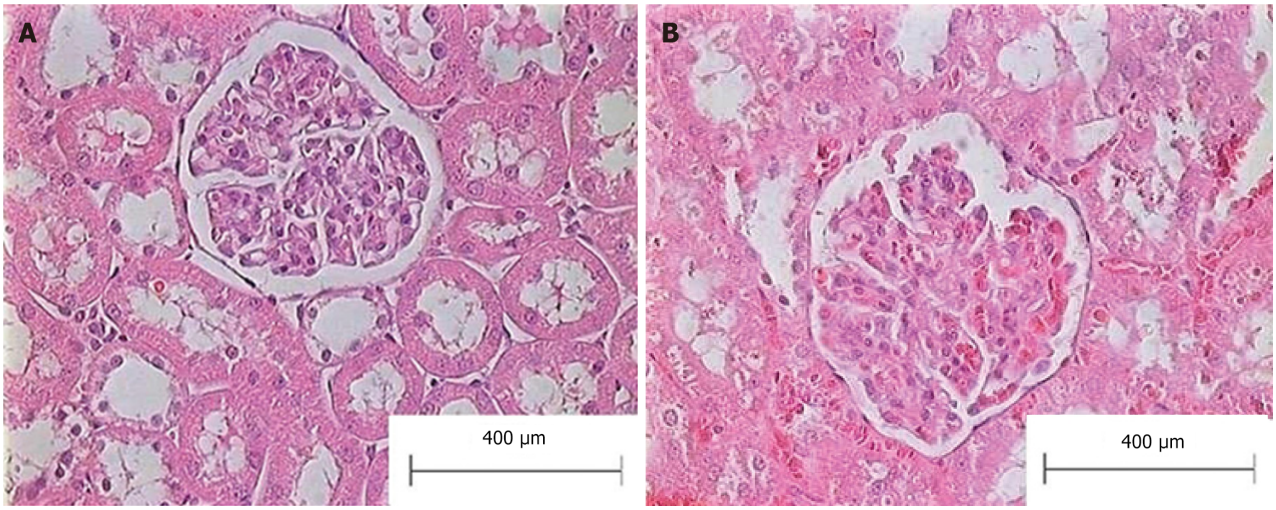

Figure 2 Representative micrographs of the renal slices (hematoxylin and eosin) from bile duct ligated and sham-operated (control) rats. A: Control group; B: Bile-duct-ligated group.

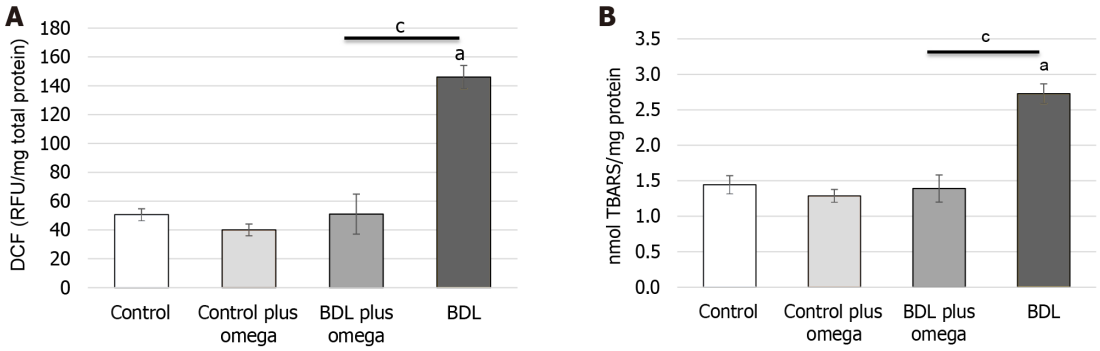

Figure 3 Effect of omega-3 on the generation of free radicals and lipoperoxidation in the kidneys of cirrhotic rats. A: *In vivo* effect of omega-3 on flow cytometry detection of reactive oxygen species in renal homogenates. Mean data for dichlorofluorescein fluorescence; B: Effect of omega-3 on lipoperoxidation measured by thiobarbituric acid reactive substances in the kidney of rats. Values represent the mean ± SD of $n = 6$ animals per group. [a]$P \leq 0.001$ *vs* control group; [c]$P \leq 0.001$ *vs* BDL with BDL plus omega group. DCF: Dichlorofluorescein; TBARS: Thiobarbituric acid reactive substances; BDL: Bile duct ligation.

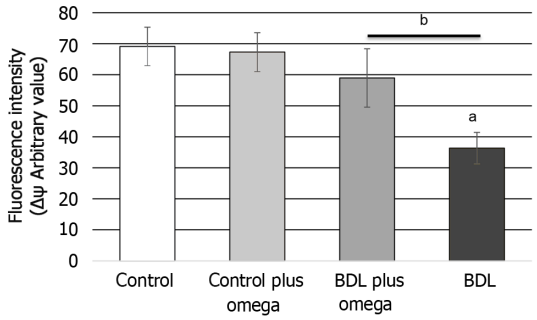

Figure 4 *In vivo* effect of omega-3 on mitochondrial membrane potential in the kidney of rats. Values represent the mean ± SD of $n = 6$ animals per group. [a]$P \leq 0.001$ *versus* control group, [b]$P \leq 0.01$ *versus* BDL with BDL plus omega group. BDL: Bile duct ligation.

DISCUSSION

Decompensated liver cirrhosis in an advanced stage is difficult to treat, as it affects several systems and organs, causing numerous complications such as PH, ascites, hepatic encephalopathy, spontaneous bacterial peritonitis, and HRS[4]. In HRS, deterioration of renal function is mainly caused by systemic circulatory dysfunction[3]. There is a significant constriction of the blood vessels that irrigate the kidneys due to splanchnic vasodilation, which increases central blood volume and interferes with renal reflex mechanisms in response to PH[7]. HRS is understood as a poor prognosis for liver disease patients, as it presents high mortality rates, especially in decompensated patients and noncandidates for liver

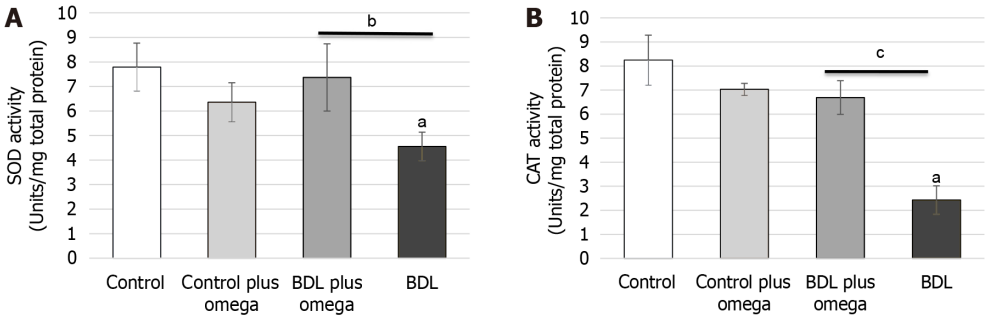

Figure 5 Effect of omega-3 on the activity of antioxidant enzymes measured in the kidneys of cirrhotic rats. A: *In vivo* effect of omega-3 on superoxide dismutase activities in the kidneys of rats. B: *In vivo* effect of omega-3 on catalase activities in the kidneys of rats. Values represent the mean ± SD of n = 6 animals per group. $^{a}P \leq 0.001$ *versus* control group, $^{b}P \leq 0.01$ and $^{c}P \leq 0.001$ *versus* BDL with BDL plus omega group. BDL: Bile duct ligation; SOD: Superoxide dismutase; CAT: Catalase.

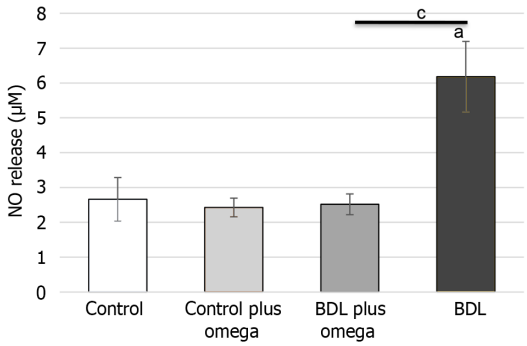

Figure 6 *In vivo* effects of omega-3 on nitric oxide production. Values represent the mean ± SD of n = 6 animals per group. $^{a}P \leq 0.001$ *versus* control group, $^{c}P \leq 0.001$ *versus* BDL with BDL plus omega group. BDL: Bile duct ligation; NO: Nitric oxide.

transplantation[6,38].

The functional nature of HRS means that improvement in renal function is expected with liver transplantation, which remains the optimal treatment of AKI-HRS whenever feasible. However, kidney recovery is not universal and depends on multiple factors, particularly the duration of kidney injury. In such cases, simultaneous liver–kidney transplantation is recommended rather than liver transplantation alone. Despite best efforts, almost 10% of patients with either acute kidney disease or chronic kidney disease who receive a liver alone may have persistent or progressive renal failure after transplantation[7]. In this context, searching for complementary treatments that can protect the kidney from the damage caused by liver cirrhosis is essential to guarantee the quality of life of the post-transplant patient.

The use of omega-3 polyunsaturated fatty acids has aroused researchers' interest in recent decades due to their various roles in treating and reducing the risk of diseases. The main types of omega-3 are eicosapentaenoic acid (EPA) and DHA, found in cold-water fish. Omega-3 can also be found in seeds and oilseeds, such as walnuts, chestnuts, flaxseeds, and sunflower seeds, in the form of linolenic acid, a precursor to the endogenous synthesis of EPA and DHA. However, this bioconversion is limited[39]. Numerous studies have highlighted the benefits of using omega-3 in cardiovascular diseases [40], diabetes[41], immune system and inflammatory modulation[42], and neurodegenerative diseases[43].

In the present study, we used animal model rats subjected to bile duct ligation for 28 d, according to the protocol by Kountouras *et al*[26], which allows the development of the clinical picture of secondary biliary cirrhosis triggered by the accumulation of bile in the liver parenchyma. In this model, from 4 wk of BDL, it is possible to identify HRS characteristics with ascites, fluid imbalance, sodium retention, and increased creatinine[10]. Renal failure observed in the chronic BDL model shares pathophysiological similarities with HRS[44].

Animal models of human diseases are important tools for studying the cellular and molecular mechanisms involved and allow the possibility of using invasive methods and testing new therapies that would be unfeasible in human patients, whether for technical or ethical reasons. From this perspective, we inquire about the benefits of omega-3 supplementation in light of the complex and difficult-to-manage scenario that HRS presents to patients affected by the disease.

In this model, we evaluated the effect of supplementation with 1 g/kg/d of omega-3 for 2 wk from the day 14 of BDL on the kidney tissue of cirrhotic rats. We observed a significant reduction in markers of oxidative stress with a decrease in the production of ROS, lipoperoxidation measured by TBARS, improvement in mitochondrial dysfunction assessed by mitochondrial electrochemical potential, and an increase in the activity of SOD and CAT.

Both EPA and DHA have a range of antioxidant and anti-inflammatory effects. Inflammation and oxidative stress are interrelated, and oxidative stress can activate inflammatory signaling pathways, while inflammation induces oxidative stress. Therefore, agents that act to reduce oxidative stress can also have anti-inflammatory action and vice versa[45].

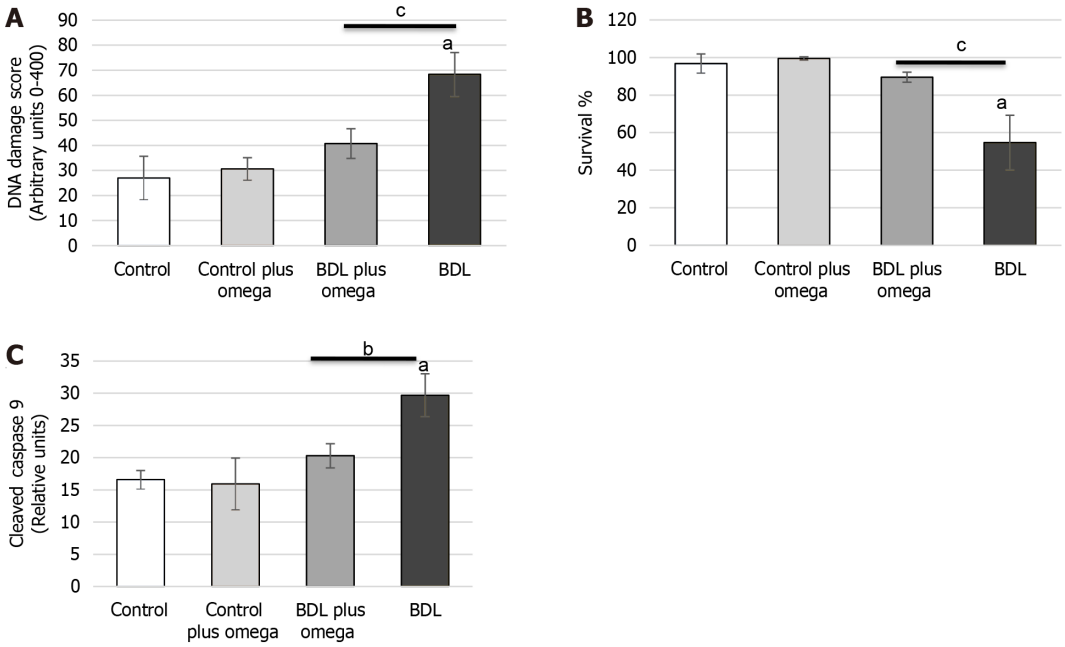

Figure 7 Effect of omega-3 on DNA damage and cell survival in the kidney of cirrhotic rats. A: *In vivo* effect of omega-3 on DNA damage in the kidneys of rats; B: Effect of omega-3 on cell viability determined by trypan blue dye-exclusion assay; C: Effect of omega-3 on caspase 9 cleaved. Values represent the mean ± SD of $n = 6$ animals per group. $^{a}P \leq 0.001$ *versus* control group, $^{b}P \leq 0.01$ and $^{c}P \leq 0.001$ *versus* BDL with BDL plus omega group. BDL: Bile duct ligation.

In subjects with cirrhosis and PH, oxidative stress plays an essential role in the pathogenesis of several complications. These complications include hyperdynamic circulation, cirrhotic cardiomyopathy, and AKI/HRS. Antioxidants can reverse or mitigate these processes and thus may have potential therapeutic effects on cardiovascular and renal abnormalities in cirrhosis[46].

Omega-3 polyunsaturated fatty acids can act as antioxidants and modulate inflammatory processes[47]. Intervention studies have demonstrated that increased intake of EPA + DHA results in increased concentrations of these fatty acids in cell membranes, which may be partly responsible for reducing membrane lipoperoxidation. Furthermore, they decrease the production of eicosanoids derived from arachidonic acid (AA)[25,48,49]. Increased omega-3 content is associated with reduced levels of other inflammatory markers, including several cytokines and chemokines, acute phase proteins, and adhesion molecules[50,51].

The use of omega-3 in an animal model of hepatitis was also able to reduce the production of ROS[52]. In contrast, in patients with advanced NAFLD, the effects of omega-3 supplementation are not evident. However, in the early stages of liver disease, omega-3 supplementation may be effective in counteracting oxidative stress and steatosis[53,54] since there is a correlation between NAFLD and depletion of polyunsaturated fatty acids[55]. In an animal model of alcoholic liver disease, omega-3 supplementation decreased steatosis and alcohol-induced liver injury through multiple mechanisms, including decreased *de novo* lipogenesis and lipid mobilization from adipose tissue, increased beta-oxidation of mitochondrial fatty acids, reduction of liver inflammation and oxidative stress[56]. The reduction in inflammation results from the well-described effects of EPA and mainly DHA in competing with AA for the delta-6-desaturase enzyme. This event forms metabolites with anti-inflammatory action, such as resolvins, maresins, and protectins. Decreased lipid accumulation in the liver is mediated through modulation of the activity of nuclear transcription factors such as peroxisome proliferator-activated receptors and sterol regulatory element-binding protein 1c. Responsive carbohydrate binding involves inflammatory pathways and liver lipid metabolism[24,25]. The use of omega-3 in liver disease improves the condition of the liver and thus reduces damage to kidney tissue since the kidney changes observed in HRS are due to complications of cirrhosis. Previous studies have already demonstrated that the use of antioxidants improves and/or protects the liver from the development of cirrhosis and reduces disease complications[57-59]. Cirrhotic animals treated with melatonin showed a reduction in hepatic transaminases in the blood, a decrease in lipoperoxidation, and an increase in SOD activity in the liver, which led to reduced liver damage[58]. Also, in the BDL cirrhosis model, melatonin treatment improved biochemical and histological parameters and liver inflammatory markers. A significant reduction in lipoperoxidation, SOD activity, and NO levels was observed[59]. In a model of severe acute hepatitis, the combination of resveratrol + ε-viniferin had a hepatoprotective effect, reducing DNA damage, exhibiting a protective role in the antioxidant pathway by altering the activities of SOD, CAT, and glutathione s-transferase, negatively regulating tumor necrosis factor (TNF)-α, cyclo-oxygenase (COX)-2, and inducible NO synthase (iNOS) and increasing interleukin (IL)-10 [57].

In a chronic kidney injury model with C57BL/6 mice treated with omega-3 for 7 d, lower oxidative stress, inflammation, and fibrosis were demonstrated compared to untreated mice[60]. It has already been shown that omega-3 derivatives, maresins, and protectins reduce inflammation and protect the kidneys in AKI[61]. In a model of AKI associated with sepsis, pretreatment with maresin-1, a lipid mediator derived from DHA, reduced the production of

malondialdehyde and improved the activity of SOD in renal tissues while inhibiting the production of ROS and protected mitochondria[62]. In a clinical trial with hemodialysis patients who received 3 g of omega-3 for 2 mo, an increase in the antioxidant enzymes SOD and glutathione peroxidase and a reduction in lipoperoxidation were observed, indicating an improvement in the antioxidant status of hemodialysis patients[63]. When added to cell membranes, polyunsaturated fatty acids act as antioxidants and regulate antioxidant signaling pathways. Mitochondrial membranes of eukaryotic cells have a high content of DHA, indicating that DHA is a fundamental phospholipid for synthesizing ATP by oxidative phosphorylation. In mitochondria, DHA acts by reducing oxidative stress and the activity of cytochrome-c oxidase (complex IV), as well as increasing the activity of manganese-dependent SOD[47,64]. The protective effect of omega-3 on cardiovascular disease is well known, and dietary supplementation has been shown to reduce mitochondrial dysfunction related to oxidative stress and endothelial cell apoptosis, an effect that occurs through an increase in the activity of endogenous antioxidant enzymes[47].

Systemic inflammation plays an important role in the pathophysiology of HRS but the exact mechanisms by which systemic inflammation leads to HRS remain to be elucidated[65].

Another important finding in our study was the reduction in NO metabolites in the kidney tissue of animals treated with omega-3. NO is considered an ambiguous molecule that can act beneficially, such as in providing vasodilation or harmfully, in situations of oxidative stress and deficiency in the antioxidant system[66]. Our research showed a significant increase in NO metabolites in the kidney tissue of cirrhotic animals, and omega-3 attenuated NO production in treated animals, highlighting an antioxidant effect of omega-3 supplementation. This effect was also observed by Bosco *et al*[67] in the lung tissue of cirrhotic animals in the hepatopulmonary syndrome model when treated with melatonin. It is known that an increase in oxidative stress induces endothelial dysfunction since ROS reduces the bioavailability of NO and increases the synthesis of more toxic oxidative species, such as peroxynitrite[68]. In cirrhosis, the presence of PH increases the release of NO through increased expression and activity of iNOS, and renal vasoconstriction, typical of HRS, also increases the NO content resulting from the activation of iNOS. In this condition, the increase in NO levels is directly related to the oxidative damage to tissues resulting from peroxynitrite formation[69]. Omega-3 supplementation reduces NO levels in kidney tissue and thus reduces the damage caused by nitrosative stress. Omega-3 stimulates the expression and activity of endothelial NO synthase (eNOS), which is one of the important effects of the cardiovascular protective action exerted by omega-3. EPA determines greater NO synthesis by increasing the activation of eNOS induced by AMP-activated protein kinase and the dissociation of eNOS from the inhibitory structure protein caveolin. eNOS activity is also stimulated by DHA, which favors the connection between eNOS and heat shock protein 90, with activation of the PKB/Akt pathway[70,71]. Treatment with omega-3 was also able to reduce the rate of DNA damage assessed by the comet assay in kidney tissue, inhibit the expression of caspase-9, and increase cell viability in the kidneys of treated cirrhotic animals. DHA pretreatment in lipopolysaccharide-stimulated bone-marrow-derived macrophages blocked caspase-1 activation and IL-1 secretion[72]. EPA treatment also reduced oxidative damage and expression of caspase-9 and caspase-3 in a mouse model of diabetic kidney injury[73]. In a vancomycin renal injury model, DHA inhibited oxidative stress and inactivated the MAPK signaling pathway, which was associated with upregulation of Bcl-2 and downregulation of caspase-9, caspase-3, cytochrome-c, p38 and C-Jun N-terminal kinase (JNK)[74]. In a model of liver injury caused by valproate, treatment with DHA prevented hepatocellular apoptosis by reducing the expression of cleaved caspase-9 and the number of positive hepatocytes by the TUNEL technique[75]. Caspase-9 acts as an apoptotic trigger stimulated by factors such as NADPH oxidase (NOX)/ROS, extracellular signal-regulated kinase (ERK)1/2, or COX-2, which leads to oxidative and inflammatory damage in liver tissue. Omega-3, in addition to its important antioxidant effect, is capable of increasing anti-inflammatory prostaglandin E3 and inhibiting IL-1 and TNF-α, in addition to negatively regulating MAPK and ERK and thereby reducing inflammation and apoptosis[75]. Using bioactive metabolites derived from EPA and DHA, such as maresins, resolvins, and protectins, has demonstrated promising results in improving many inflammatory diseases[76]. In a model of AKI associated with sepsis, pretreatment with maresin-1, a lipid mediator derived from DHA, inhibited the expression of Bax and cleaved caspase-3 while increasing the expression of Bcl-2 in renal tissues. This effect appears to occur through inhibiting the NOX4/ROS/NF-κB p65 signaling pathway[64]. Maresin-1 also improved inflammation, reduced necrotic areas, and increased cell proliferation, as assessed by the mitotic activity index and Ki-67 expression in the livers of NAFLD animals[77]. Pretreatment with RvD1, another lipid mediator derived from DHA, attenuated apoptosis induced by endoplasmic reticulum stress and also decreased caspase 3 activity in HepG2 cells, an effect mediated through the JNK pathway[78].

In summary, the use of omega-3 treatment for 2 wk in cirrhotic animals from day 14 of BDL showed protective effects on renal tissue, as we demonstrated an antioxidant effect through the reduction in the formation of ROS, decreased lipoperoxidation measured by TBARS, decreased in NO metabolites. Furthermore, there was improvement in mitochondrial electrochemical potential and an increase in the activity of the antioxidant enzymes SOD and CAT in the kidneys of treated cirrhotic animals. We also demonstrated the protection of kidney tissue by reducing DNA damage, apoptosis, and cell death in animals with BDL treated with omega-3.

CONCLUSION

The treatment of HRS remains a challenge. Novel therapies such as serelaxin (recombinant human relaxin-2) that increase renal blood flow, reduce renal vascular resistance, and reverse endothelial dysfunction[79] have not been evaluated in HRS[7]. The use of pentoxifylline, a phosphodiesterase inhibitor with anti-TNF-α activity and anti-inflammatory effect, is well established in patients with AKI-HRS. However, it does not show an added benefit compared with the standard of care alone (midodrine, octreotide, and albumin)[80]. Translational experimental models of HRS are needed to fill this gap

and, hopefully, help identify novel targets for potential drug development. Meanwhile, using substances with protective antioxidant and anti-inflammatory effects can help protect the kidney from the deleterious effects of cirrhosis.

Our results suggest that omega-3 protects kidney tissue by improving oxidative and inflammatory stress parameters and protecting against cellular damage triggered in the kidney by liver cirrhosis. This protective effect may be necessary for maintaining the integrity of renal tissue in patients with advanced liver disease waiting for liver transplantation.

FOOTNOTES

Author contributions: Porawski M and Saffi J designed and coordinated the study; Duailibe JBB, Viau CM, performed the experiments, acquired and analyzed data; Duailibe JBB, Viau CM, Fernandes SA and Porawski M analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Deendayal D Mishra, Pramod K Maurya, Swasti Tiwari

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Abstract

BACKGROUND

Kidney disease is a severe complication of diabetes that often leads to end-stage renal disease. Early diagnosis is crucial for prevention or delay. However, the current diagnostic methods, with their limitations in detecting the disease in its early stages, underscore the urgency and importance of finding new solutions. miRNAs encapsulated inside urinary exosomes (UEs) have potential as early biomarkers for kidney diseases. The need for reference miRNAs for accurate interpretation currently limits their translational potential.

AIM

To identify consistently expressing reference miRNAs from UEs of controls and patients with type 2 diabetesmellitus (T2DM) and biopsy-confirmed kidney diseases.

METHODS

miRNA profiling was performed on UEs from 31 human urine samples using a rigorous and unbiased method. The UEs were isolated from urine samples collected from healthy individuals (n = 6), patients with T2DM (n = 13), and T2DM patients who also had kidney diseases (including diabetic nephropathy, n = 5; membranous nephropathy, n = 5; and IgA nephropathy, n = 2) through differential ultracentrifugation. After characterizing the UEs, miRNA expression profiling using microarray technology was conducted.

RESULTS

Microarray data analysis identified 14 miRNAs that were consistently expressed in UEs from 31 human samples, representing various kidney conditions: diabetic controls, diabetic nephropathy, membrane nephropathy, IgA nephropathy, and healthy controls. Through *in silico* analysis, we determined that 10 of these miRNAs had significant potential to serve as reference genes in UEs.

CONCLUSION
We identified uniformly expressing UE miRNAs that could serve as reference genes kidney disease biomarkers.

Key Words: miRNA; Microarray; Urinary exosomes; Diabetic nephropathy; Type 2 diabetes mellitus; Kidney disease

Core Tip: Early prevention and detection of end-stage renal disease is currently inadequate. miRNAs found in urinary exosomes (UEs) may serve as early biomarkers for kidney diseases. However, the lack of reference miRNAs for normalization makes it difficult to interpret the data from UEs. In this study, we profiled the miRNAs in UEs from 31 human urine samples and identified a set of consistently expressed miRNAs that could be utilized as reference gene biomarkers for kidney disease.

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INTRODUCTION

Kidney disease is a severe complication of diabetes[1], and presents clinical hallmarks of diabetic nephropathy. This progression includes an initial phase of glomerular hyperfiltration, increasing albuminuria, and hypertension, ultimately leading to a gradual decline in renal function and ending in end-stage renal disease (ESRD) after 5–15 years. Patients with ESRD are left with the only choice of renal replacement therapies, which is an economic burden and results in poor quality of life. However, timely diagnosis could prevent or, at least, delay this debilitating condition. The existing diagnostic methods in clinical use are limited in their ability to detect the disease at its early stage. However, the discovery of urinary exosomes (UEs), rich in possible biomarkers for kidney disease, offers a promising source for diagnosis, instilling hope for improved early detection and intervention.

Extracellular vesicles (EVs) are membrane vesicles released by almost every cell of the body into the extracellular fluid, such as blood, urine, semen, saliva, breast milk, tears, and bile[1-8]. In the urine, the EVs originate from the cells lining the urinary tract and the entire nephron lumen. The EVs, particularly the nanosized ones known as exosomes, and in the case of urine specifically referred to as UEs, are thought to reflect the content of their originating cell as they originate in the late endosomal compartment by the inward budding of multivesicular bodies. They play an essential role in mediating cellular communication, primarily by transferring their nucleic acid content (mRNA and miRNA)[9-11]. Thus, the EVs are of scientific interest for their diagnosis and therapeutic potential, including drug delivery and targeted therapy. UEs stand out for their high diagnostic value in kidney disease biomarker discovery, making them a promising area of research[2,12-15]. However, optimal normalization is a significant challenge.

Albumin excretion over 24 hours (usually g/day or μg/min) is a classical normalization method for urine-based biomarkers. Still, it has a limitation of total dependency on the urine volume, which is generally unstable and changes governed by body size, hydration status, and other factors[16,17]. Normalization using creatinine is the standard method; however, the creatinine excretion rate varies in certain conditions, such as precariousness in acute kidney injury[18-20]. The sizing and counting of the number of UEs in whole urine samples have also been recommended but require sophisticated, expensive equipment[21]. We propose that a normalization gene or set of genes (miRNAs) with abundant expression, which do not change with a disease condition or type and do not require costly equipment for analysis, should be used for normalization. In 2017, Lange *et al*[22] performed RT-PCR analysis of a few selected miRNAs in UEs isolated from urine samples of chronic kidney disease patients and found miR-16 among them as an endogenous reference. The present study used an unbiased approach to determine the potential reference genes for human UE data correction in kidney disease studies. This involved collecting urine samples from type 2 diabetes mellitus (T2DM) patients with/without kidney disease and from healthy subjects, and analyzing the UE miRNA expression data to determine stably expressed genes among the three groups.

MATERIALS AND METHODS

Study design

The current study focused on identifying the reference miRNAs in UEs. The study was approved by the Institutional Ethics Committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences (Ref No. 2021-194-PhD-EXP-41). The flow chart shows step-by-step methods and analysis (Figure 1).

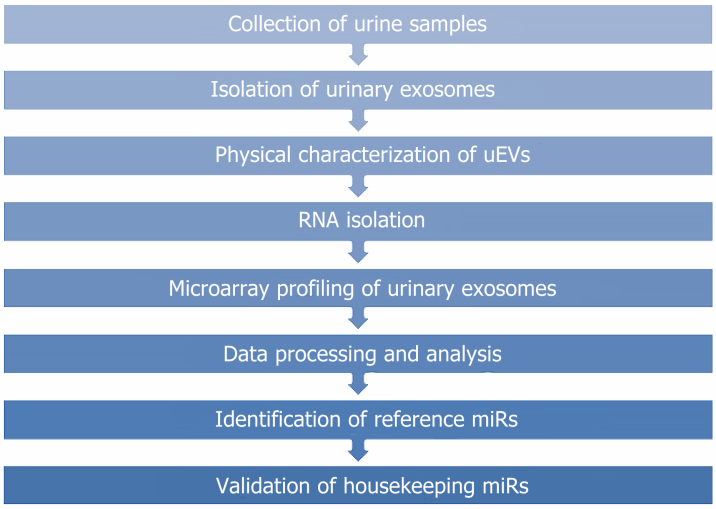


Figure 1 Flow chart for selecting housekeeping miRNAs. miRs: miRNAs.

Urine sample collection and processing

Urine samples (first morning) were collected from patients with diabetes with or without kidney disease (n = 12 or 13/group). The subjects had one of the following biopsy-proven kidney disease subtypes; diabetic nephropathy (n = 5), membranous nephropathy (n = 5), or IgA nephropathy (n = 2). Urine samples were collected from kidney disease patients at the Department of Nephrology, Sanjay Gandhi Post Graduate Institute of Medical Sciences-Lucknow, and Christian Medical College-Vellore, India. Urine samples were also collected from six healthy subjects. Urine samples were collected from all the subjects after obtaining written informed consent. These samples underwent low-speed centrifugation (1000 × g for 10 min at 4°C) to eliminate cellular debris then stored at 80°C with the addition of a protease inhibitors cocktail (1.67 mL of 100 mmol/L NaN_3, 2.5 mL of 11.5 mmol/L 4-[2-aminoethyl] benzenesulfonyl fluoride, and 50 l of 1 mmol/L leupeptin). Table 1 shows the number of urine samples collected from three groups: T2DM patients without kidney disease; T2DM patients with kidney disease; and healthy subjects without T2DM or kidney disease.

Isolation of UEs from urine

UEs were isolated from a 10-mL aliquot of the diluted urine as previously described[23], using high-speed centrifugation following an initial low-speed spin to remove whole cells and debris.

Characterization of UEs

After isolation, UEs were physically characterized by nanoparticle tracking analysis (NTA) with the NanoSight NS300 (Malvern Instruments Ltd., UK) at the Central Analytical Research Facility of the Indian Institute of Toxicological Research, Lucknow. Additionally, exosomal proteins from urine samples were analyzed *via* immunoblotting with antibodies against exosome-specific markers (CD9, CD63 and CD81) from Abcam (Cambridge, MA, USA). All images were captured using the ChemiDoc Imaging System (Universal Hood III, Bio-Rad, Hercules, CA, USA).

RNA isolation

Total RNA was extracted from the UE pellet using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA). The concentrations of total RNA were measured with a bioanalyzer (Agilent, Santa Clara, CA, USA)/spectrophotometer (NanoDrop™ 2000/2000c).

Microarray profiling

Microarray analysis was conducted using the Affymetrix Human miRNA 4.0 Array (Affymetrix-GeneChip™ miRNA 4.0 Array; Thermo Fisher Scientific, Waltham, MA, USA). Biotin-labelled RNA was created using the Affymetrix® FlashTagTM Biotin HSR RNA Labelling Kit (Thermo Fisher Scientific). Approximately 130 ng of total RNA underwent poly-A tailing at the 3' end, followed by the attachment of a biotin-labeled 3DNA molecule *via* DNA ligase. The biotin-labeled RNA samples were hybridized to GeneChip miRNA 4.0 arrays in an Affymetrix® Oven 455 (Thermo Fisher Scientific) at 48°C for 18 h with 60 rpm rotation. After hybridization, the miRNA 4.0 arrays were washed and stained using the GeneChip® Hybridisation, Wash, and Stain Kit (Thermo Fisher) in Fluidics Station 450 (Thermo Fisher Scientific). The hybridized targets on the arrays were stained with streptavidin–phycoerythrin from the kit and detected using 3000 7G scanner (Thermo Fisher Scientific). Transcriptome Analysis Console software was used for data analysis.

Data processing and analysis for housekeeping miRNAs

The raw expression profile data were obtained as CEL files and were further processed using Affymetrix® Transcriptome Analysis Console software (version 4.0, Thermo Fisher Scientific). Background correction, quantile normalization, and data normalization with the expression console were done. After normalization of the microarray data, the expression of

Table 1 Number of subjects enrolled for microarray profiling

Groups	Kidney conditions	Group	Urine samples
Group 1	T2DM patients without kidney disease	Diabetic control	13
Group 2	T2DM patients with kidney disease	Diabetic nephropathy	5
		Membranous nephropathy	5
		IgA nephropathy	2
Group 3	Healthy subjects without T2DM or kidney disease	Healthy control	6

T2DM: Type 2 diabetes mellitus.

miRNA derived from UEs was analyzed by evaluating mean and SD instead of consistency among all 31 expression profiles categorized into three groups, as shown in Table 1. Mean and SD were evaluated for selecting homogeneously expressing miRNAs using one way ANOVA in SPSS software (IBM Corp. Version 20.0. Armonk, NY, USA). The absolute deviation of miRNA expression among groups was selected using the range value. The range value of 0.8 was chosen to filter out the genes having significant, consistent expression in all 31 urine samples. The clusters (http://biit.cs.ut.ee/clustvis/) software was utilized to draw the clustering heatmap of these identified common signatures[24].

Functional annotation and enrichment of miRNAs

The functional enrichment analysis of the evenly expressing miRs was performed using the miRNA Enrichment Analysis and Annotation Tool (miEAA 2.0; https://www.ccb.uni-saarland.de/mieaa2), and a miRNA-centric network visual analytics platform (miRNet 2.0; https://www.mirnet.ca). The miEAA 2. 0 provided the annotation of miRNAs in various categories such as related pathway, disease, chromosomal location, site of expression, miRNA–transcription factor (TF) interactions, family, and PubMed annotation[25]. The miRNet 2.0 generated an allied network of centric miRNA with TFs, target genes (from experimentally validated miRTarBase v8.0 database), associated disease and epigenetic modifiers [26]. For both tools, false discovery rate < 0.05 was considered significant for selecting annotation features.

Statistical analysis

GraphPad Prism 8 was used to prepare the graph by calculating significant mean values from tabular data by using one-way ANOVA.

RESULTS

Demographics of subjects

The demographic details of subjects with five different kidney conditions *i.e.*, diabetic controls, diabetic nephropathy, membranous nephropathy, IgA nephropathy, and healthy controls (Table 2), from whom, urine samples were collected for isolation of UEs. A total of 31 urine samples were collected, of which 13 were from diabetic controls, five each from diabetic nephropathy or membranous nephropathy, two from IgA nephropathy, and six from healthy controls. Patients with membranous nephropathy had highest mean age, while lowest duration of diabetes. Patients with membranous nephropathy and healthy controls were overweight with average BMI > 29.9.

Detection of UEs in urine samples

The isolated UEs were physically characterized using NTA. Figure 2A presents NTA plots depicting the average concentration (particles/mL) and size of vesicles isolated from human urine samples, with peak sizes at 70 nm and 191 nm corresponding to exosomes. In Figure 2B, the concentration distribution clearly shows that the small vesicles (below 200 nm) were more abundant in our preparation compared to moderate or large particles. Additionally, vesicles ranging between 30 and 150 nm in size had the highest intensity. A total of 40 μg of UE protein showed the presence of exosome-specific marker proteins, CD9, CD63 and CD81, using western blotting (Figure 2C).

Identification of housekeeping miRNAs

A total of 14 miRNAs showed consistent expression across all groups, both healthy and diseased (Table 3). Figure 3A illustrates the uniform expression of these 14 miRNAs (considered housekeeping genes) across the three groups, while Figure 3B displays a heatmap of these miRNAs.

Validation of housekeeping miRNAs

The 14 identified housekeeping miRNAs were further analyzed and annotated using the miEAA 2.0 server. This explored their links to diseases, associations with TFs, interactions with mRNAs or genes, and their sites of expression. Among the 14 reference miRNAs, seven showed associations with various diseases according to the Human MicroRNA Disease Database, and 13 were found to interact with different TFs based on data from TransmiR (a transcription factor–miRNA

Table 2 Demographic and anthropometric details of subjects enrolled in the study

Serial number	Variable	Diabetic control	Diabetic nephropathy	Membranous nephropathy	IgA nephropathy	Healthy control	*P* value
1	Sample size (*n*)	13	05	05	02	06	NA
2	Age (yr), mean ± SD	45.11 ± 7.65	47.11 ± 12	51 ± 3.8	49 ± 1.41	46 ± 10.79	0.04
3	Gender (male/female)	8/5	3/2	4/1	2/0	3/3	NA
4	Duration of DM (yr), mean ± SD	10± 6.08	6 ± 5	2.4 ± 2	5.12 ± 6.9	NA	0.06
5	BMI (kg/m^2), mean ± SD	24 ± 3.64	24 ± 3.64	26.6 ± 6	23 ± 5.23	26 ± 7.04	0.09
6	Medical condition	DM or DM & HTN both	DM or DM & HTN both	DM or HTN or DM & HTN both	IgA nephropathy	NA	NA

One-way ANOVA was used to compare quantitative variables between different disease groups wherever applicable. DM: Diabetic mellitus; BMI: Body mass index; HTN: Hypertension; IgA: Immunoglobulin A; NA: Not available.

Table 3 Table displaying housekeeping miRNAs and their mean expression intensity in urinary exosomes across five kidney conditions (control and disease)

ID	Transcript ID	Healthy control	Diabetic control	Diabetic nephropathy	Membranous nephropathy	IgA nephropathy
20536748	has-mir-4479	2.036667	2.024615	2.04	1.93	1.985
20537225	hsa-mir-5703	2.536667	2.173077	2.85	2.43	2.715
20534637	hsa-mir-320a	2.295	2.558462	2.026	2.562	2.495
20535940	hsa-mir-320d-2	3.778333	4.185385	3.65	3.536	3.37
20504391	hsa-miR-638	9.026667	8.643077	9	8.762	9.205
20536665	hsa-mir-4417	4.183333	4.378462	3.932	3.504	3.97
20536810	hsa-mir-4530	2.996667	2.891538	2.886	2.468	2.525
20536248	hsa-mir-3153	2.561667	3.033846	2.242	2.53	2.58
20522537	hsa-miR-5787	9.41	9.218462	9.228	8.386	8.77
20518446	hsa-miR-3921	2.045	2.076923	2.46	2.12	1.395
20536956	hsa-mir-4722	2.78	2.642308	2.768	2.358	2.49
20536702	hsa-mir-4449	2.178333	1.791538	2.69	2.364	2.925
20537581	hsa-mir-6836	2.701667	2.528462	2.566	2.272	2.025
20515578	hsa-miR-3157-3p	2.071667	2.483846	1.772	2.256	1.44

regulation database) during enrichment and annotation analysis.

The predicted tissue-specific expression of these 14 reference miRNAs indicated that 12 of them were expressed in kidney tissue, as illustrated in the heatmap (Figure 4A). Additionally, the Word cloud analysis confirmed the kidney as the primary site of expression for these housekeeping miRNAs (Figure 4B).

The fully annotated miRNA-centric network has been formed with the 14 housekeeping miRNAs with enriched features such as interacting miRNA, mRNA/protein, and their corresponding disease annotation (with a total of 3633 nodes and 5105 edges) (Figure 5A). The constructed functional network contained 18 miRNAs (13 native and five enriched), their interacting 3513 gene partners, and 102 disease annotations corresponding to the miRNAs and their interactions.

With 3633 nodes and 5105 edges, the above network was complex for analysis of the enrichment of housekeeping miRNAs. Therefore, a subnetwork or module from the above network containing 18 enriched housekeeping miRNAs, their common interactors, and corresponding diseases was extracted from the complex network. The complete function annotation of miRNA is shown in Figure 5B.

The analysis of the above-selected module showed the involvement of only two housekeeping miRNAs, hsa-mir-638 and hsa-mir-320a, in kidney-related diseases. hsa-mir-638 was associated with diabetic nephropathy, lupus vulgaris, breast neoplasms, and hepatocellular carcinoma; whereas hsa-mir-320a was found in diabetic retinopathy, T2DM,

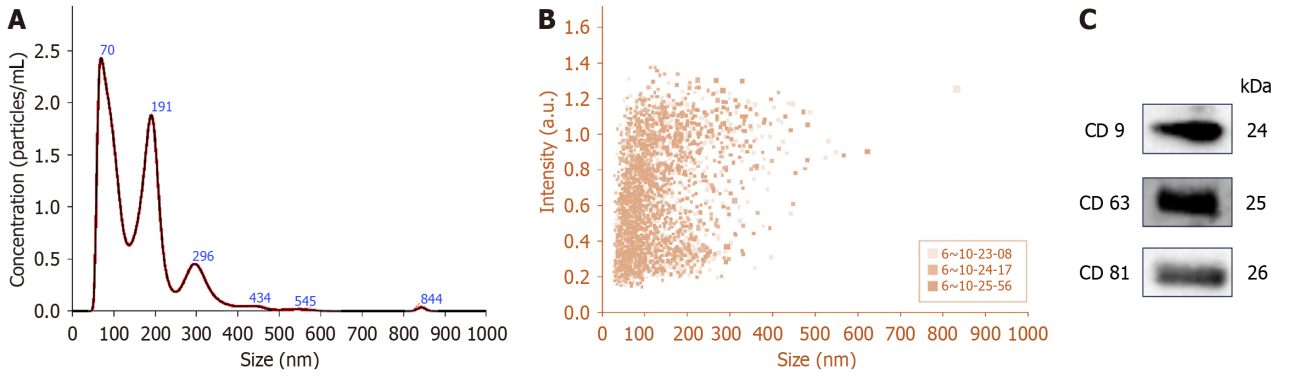


Figure 2 Characterization of urinary exosomes. A: Representative nanoparticle tracking analysis of urinary exosomes (UEs) showing exosome concentration (particles/mL)/size in pellets; B: Scattering distribution (intensity/size) profile; C: Representative immunoblot showing the presence of exosome-specific marker proteins, CD9, CD63 and CD81 in the UE protein samples. UEs were isolated from human urine samples *via* differential ultracentrifugation methods.

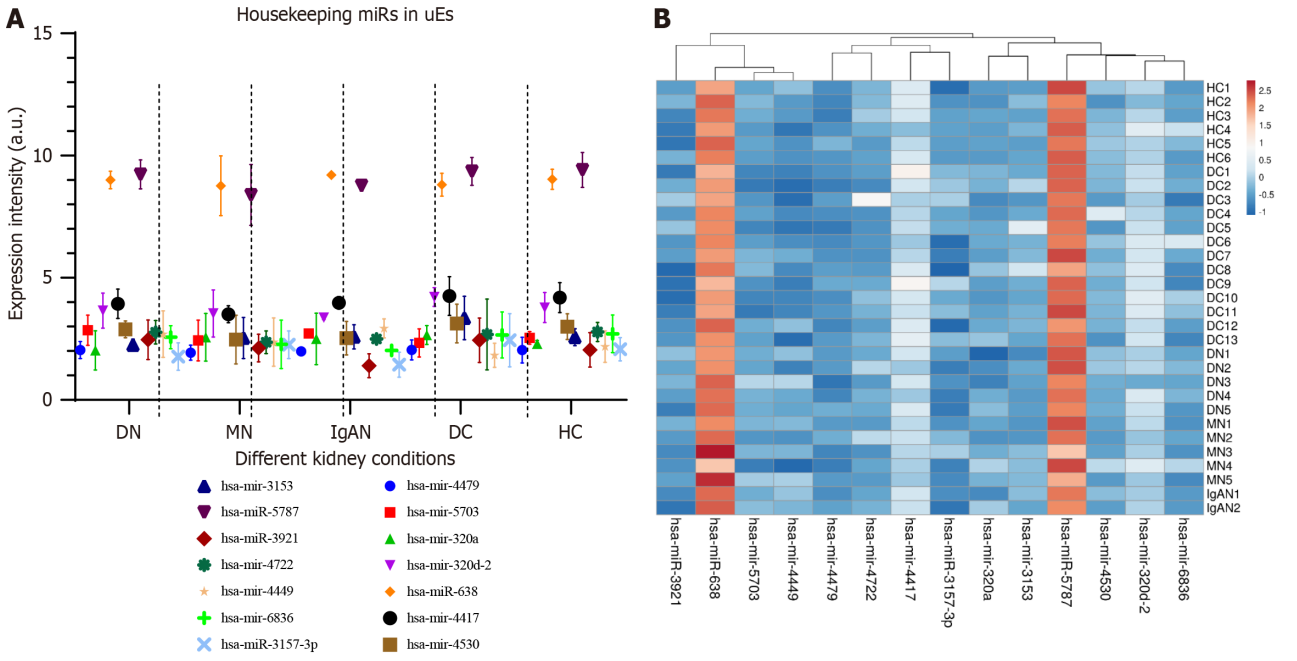


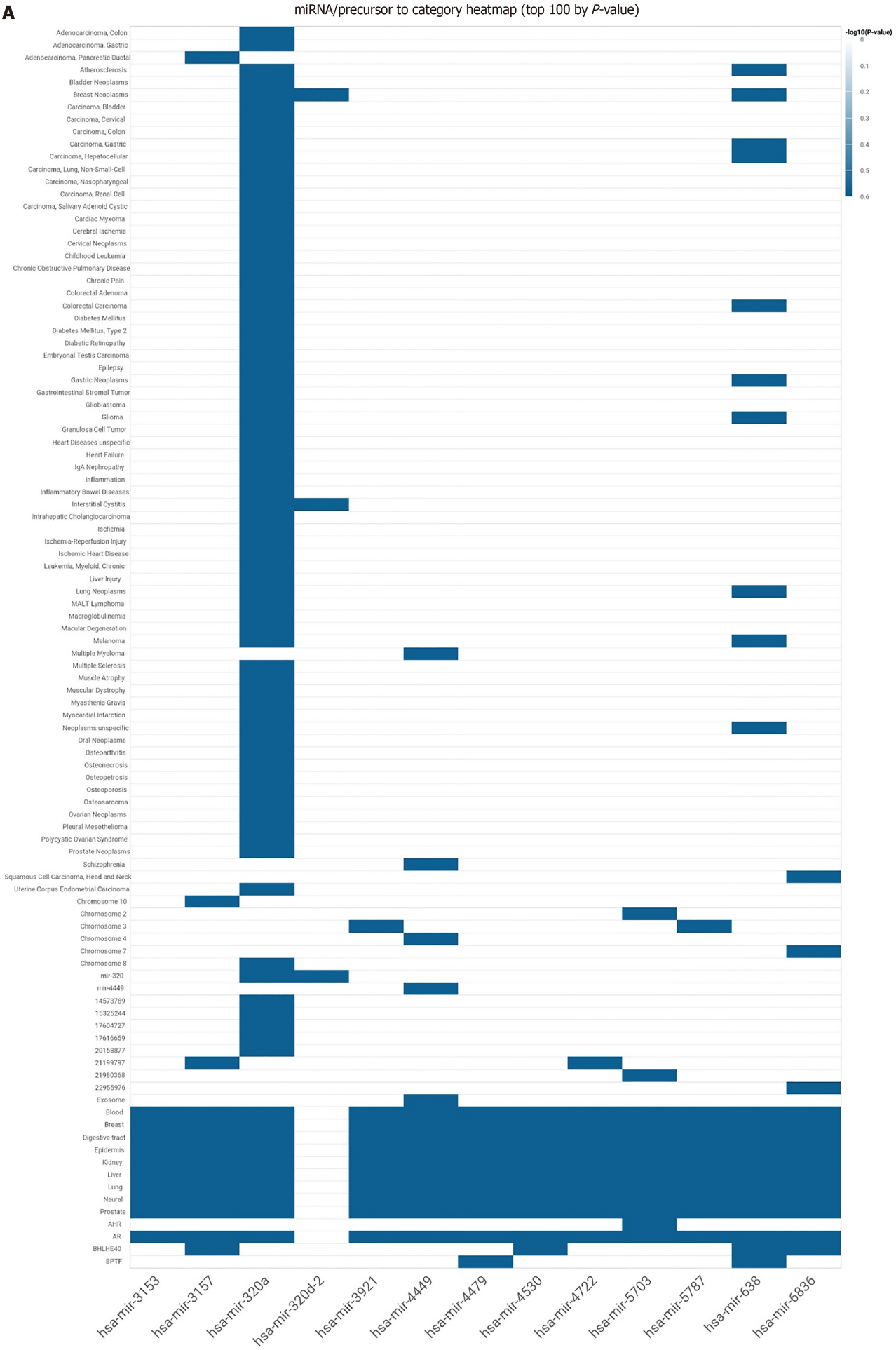
Figure 3 Expression of housekeeping miRNAs in different kidney conditions. A: Housekeeping miRNAs depicting stable expression among different kidney conditions. Error bars represent the SEM of five different conditions; B: Heat map showing the expression of 14 homologous miRNA signatures among 31 samples of different kidney conditions.

bladder neoplasms, breast neoplasms, squamous cell carcinoma, and pancreatic cancer.

DISCUSSION

The translational potential of UEs as a rich source of kidney disease biomarkers is widely recognized. However, this potential of urine EVs has been limited due to the absence of optimal reference genes (miRNAs) for accurate data normalization[22,27-29]. In a novel approach, our current study aimed to identify housekeeping/reference genes for studying kidney-disease-associated gene expression in UEs.

We identified 14 potential reference miRNAs that were expressed uniformly in the microarray profile of 31 human urinary EV samples from subjects with T2DM with or without kidney disease and healthy controls. The genes with minimal dispersion in their fold expression, from the mean value obtained from all 31 samples, were considered uniformly expressed genes. The shortlisted genes with SD < 0.8 were taken as uniformly expressed genes. A high throughput unbiased approach has not been done to identify reference/housekeeping miRNA in UEs for kidney disease studies. Only a handful of studies attempted to find potential reference genes from a targeted or shortlisted set of genes [22,30]. Our thorough approach ensured the reliability of our findings.


A
miRNA/precursor to category heatmap (top 100 by P-value)
-log10(P-value)
0
0.1
0.2
0.3
0.4
0.5
0.6
Adenocarcinoma, Colon
Adenocarcinoma, Gastric
Adenocarcinoma, Pancreatic Ductal
Atherosclerosis
Bladder Neoplasms
Breast Neoplasms
Carcinoma, Bladder
Carcinoma, Cervical
Carcinoma, Colon
Carcinoma, Gastric
Carcinoma, Hepatocellular
Carcinoma, Lung, Non-Small-Cell
Carcinoma, Nasopharyngeal
Carcinoma, Renal Cell
Carcinoma, Salivary Adenoid Cystic
Cardiac Myxoma
Cerebral Ischemia
Cervical Neoplasms
Childhood Leukemia
Chronic Obstructive Pulmonary Disease
Chronic Pain
Colorectal Adenoma
Colorectal Carcinoma
Diabetes Mellitus
Diabetes Mellitus, Type 2
Diabetic Retinopathy
Embryonal Testis Carcinoma
Epilepsy
Gastric Neoplasms
Gastrointestinal Stromal Tumor
Glioblastoma
Glioma
Granulosa Cell Tumor
Heart Diseases unspecific
Heart Failure
IgA Nephropathy
Inflammation
Inflammatory Bowel Diseases
Interstitial Cystitis
Intrahepatic Cholangiocarcinoma
Ischemia
Ischemia-Reperfusion Injury
Ischemic Heart Disease
Leukemia, Myeloid, Chronic
Liver Injury
Lung Neoplasms
MALT Lymphoma
Macroglobulinemia
Macular Degeneration
Melanoma
Multiple Myeloma
Multiple Sclerosis
Muscle Atrophy
Muscular Dystrophy
Myasthenia Gravis
Myocardial Infarction
Neoplasms unspecific
Oral Neoplasms
Osteoarthritis
Osteonecrosis
Osteopetrosis
Osteoporosis
Osteosarcoma
Ovarian Neoplasms
Pleural Mesothelioma
Polycystic Ovarian Syndrome
Prostate Neoplasms
Schizophrenia
Squamous Cell Carcinoma, Head and Neck
Uterine Corpus Endometrial Carcinoma
Chromosome 10
Chromosome 2
Chromosome 3
Chromosome 4
Chromosome 7
Chromosome 8
mir-320
mir-4449
14573789
15325244
17604727
17616659
20158877
21199797
21980368
22955976
Exosome
Blood
Breast
Digestive tract
Epidermis
Kidney
Liver
Lung
Neural
Prostate
AHR
AR
BHLHE40
BPTF
hsa-mir-3153
hsa-mir-3157
hsa-mir-320a
hsa-mir-320d-2
hsa-mir-3921
hsa-mir-4449
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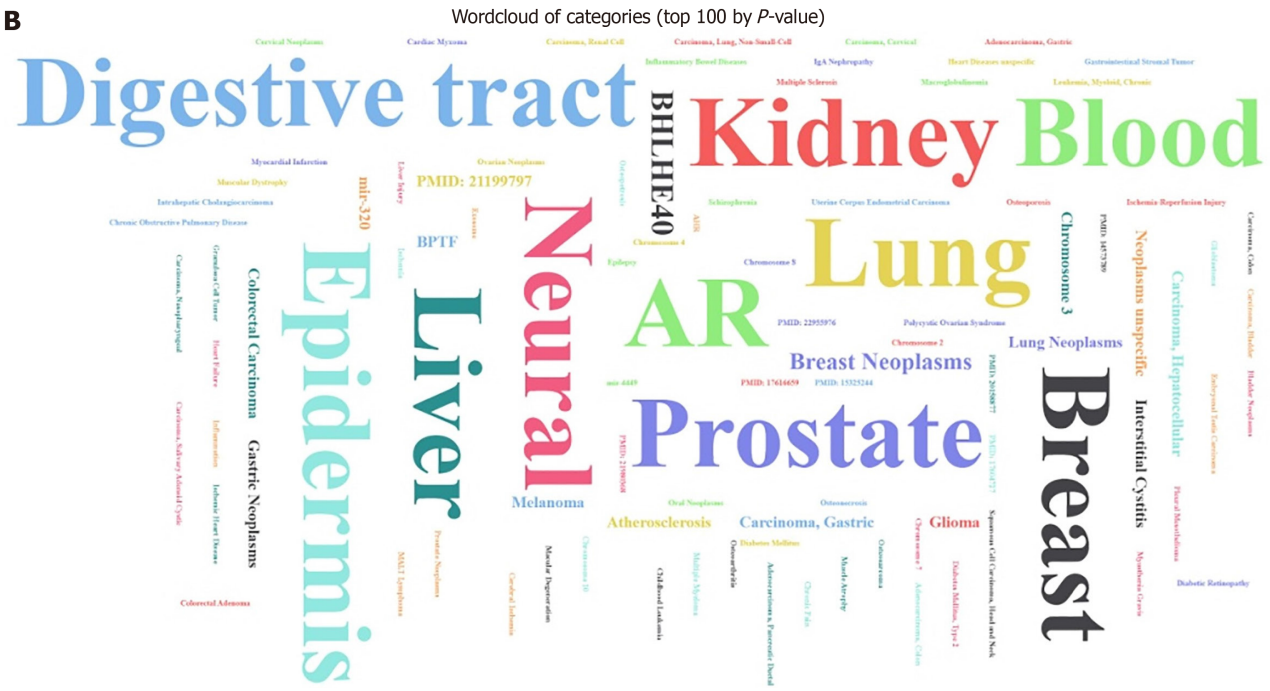

Figure 4 Enrichment analysis and annotation of housekeeping miRNAs. A: Heatmap displaying the top 100 local interactions of housekeeping miRNAs; B: Word cloud analysis displaying the top 100 sites of expression annotations for housekeeping miRNAs, based on a *P* value threshold < 0.05.

Out of the 14 potential reference genes identified in our study, we conducted a comprehensive *in silico* analysis. We found that renal expression or interactions were not found for miR-4417 and miR-320d-2. Also, the functional enrichment and annotation suggested the association of miR-320a and miR-638 with diabetic nephropathy, T2DM and diabetic retinopathy (Figure 2B). Therefore, we omitted miR-4417, miR-320d-2, miR-320a and miR-638 from our list of potential reference gene for kidney disease UE data normalization. The *in silico* analysis of the remaining 10 miRNAs showed an association of miR-4530 miR-6836 with various cancers such as biliary tract carcinoma, breast carcinoma, and head and neck squamous cell carcinoma. For the other eight miRNAs, no information was available for the pathways or disease association. All these miRNAs were reported to have a strong expression in kidney tissues. It would be interesting to determine a role for these novel miRNAs by wet laboratory experiments; however, this was beyond the scope of our present study. We did not find any published report for any of these 10 reference miRNAs identified in our study.

Our study, while comprehensive, was not without limitations. We acknowledge that our sample size was limited and we did not test in different cohorts. Moreover, validating the findings in samples from more than one time point of the study through longitudinal studies would have strengthened the findings.

The existing strategies for normalization of UE genes by RT-PCR include miRNAs (such as miR-16) reference genes for disease controls or non-disease controls[22]. However, whether these genes remain stably expressed in different kidney diseases has not been studied. In our study we included different kidney disease types, and used biopsy-proven disease samples, which ensured the endogenous expression of the reported reference genes, at least in major types of kidney disease. Several studies focusing on kidney disease have used spike-in controls for RT-PCR data normalization. These controls could be a better way to address any technical issues with the reactions; however, amplification bias, which is assumed to be gene specific, cannot be addressed by spike-in normalization[31].

CONCLUSION

Overall, we identified 10 miRNAs that were consistently expressed in different kidney diseases, and had high expression levels in kidney tissue. These miRNAs showed similar expression levels in UEs from patients with T2DM, regardless of whether they had kidney disease, as well as in healthy individuals. Therefore, these miRNAs could serve as ideal reference genes for analyzing and normalizing data on UE miRNAs by RT-PCR in studies related to kidney disease. After confirmation in a more extensive set of samples, the multiple gene panels reported here could provide a more accurate interpretation and be preferred to a single reference gene for better accuracy.

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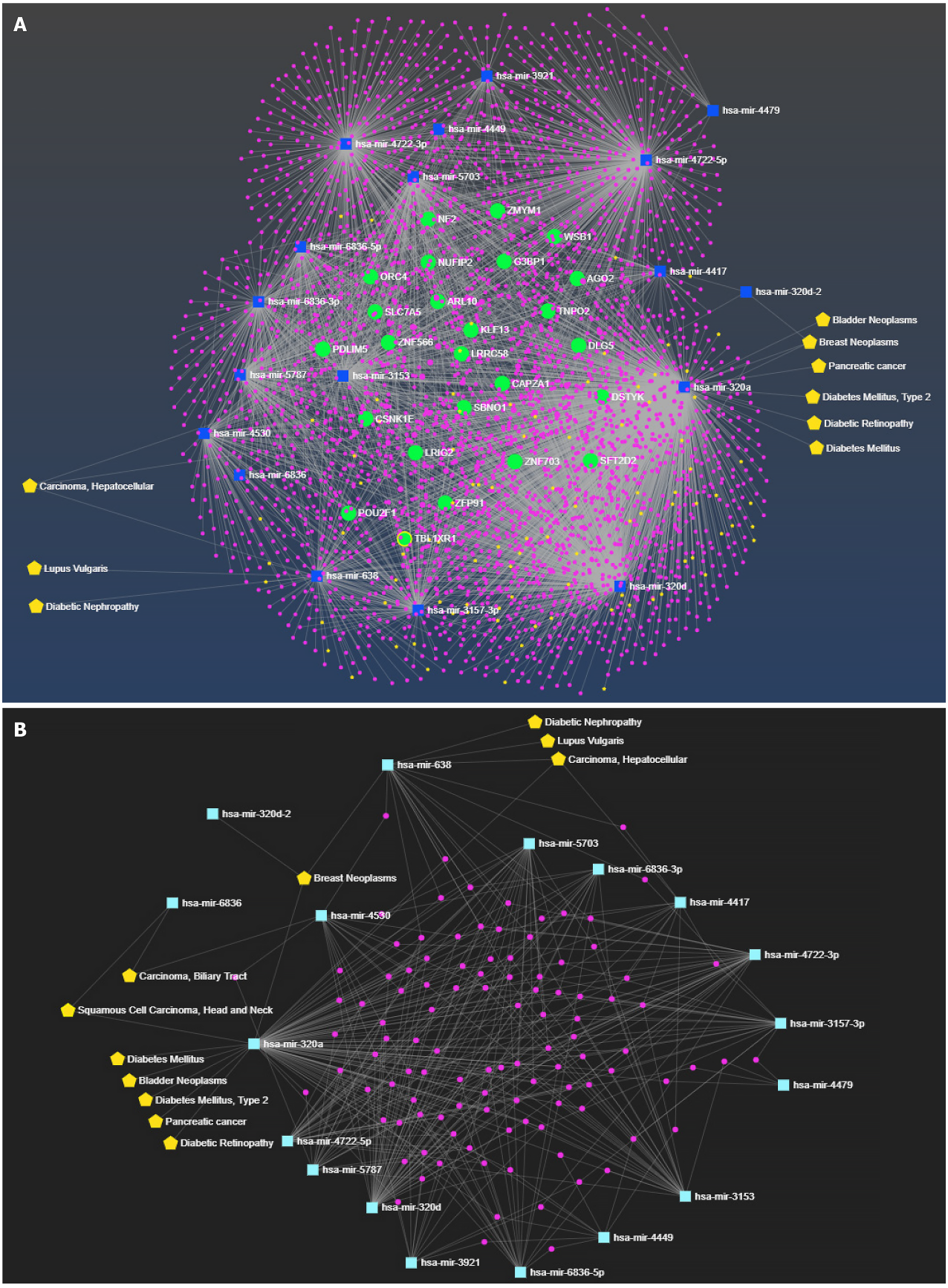

Figure 5 miRNA-centric network of miRNAs showing interactor gene, and diseases associations. A: Allied network of miRNA and its interactors with disease annotation. Green color nodes represent the interactor gene, blue nodes represent the respective miRNAs, and yellow color nodes represent the associated disease conditions; B: Subnetwork of miRNA and its common interactors with disease annotation. Pink color nodes represent the interactor gene, light blue color node shows the respective miRNAs and yellow color nodes represent the associated disease conditions.

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FOOTNOTES

Author contributions: Mishra DD performed the experiments, reviewed the literature, analyzed the data, and wrote the manuscript; Maurya PK performed the experiments, analyzed the data, and wrote the manuscript; Tiwari S designed the study, analyzed the data, and wrote and revised the manuscript; All the authors have read and approved the final version of the manuscript.

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Institutional review board statement: The study was reviewed and approved by the Sanjay Gandhi Postgraduate Institute of Medical Sciences Institutional Review Board [Ref No. 2021-194-PhD-EXP-41].

Informed consent statement: Participants were enrolled in the study after written informed consent.

Conflict-of-interest statement: All the authors declare that they have no conflict of interest.

Data sharing statement: Data used in the manuscript is available from the corresponding author at Tiwaris@sgpgi.ac.in.

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