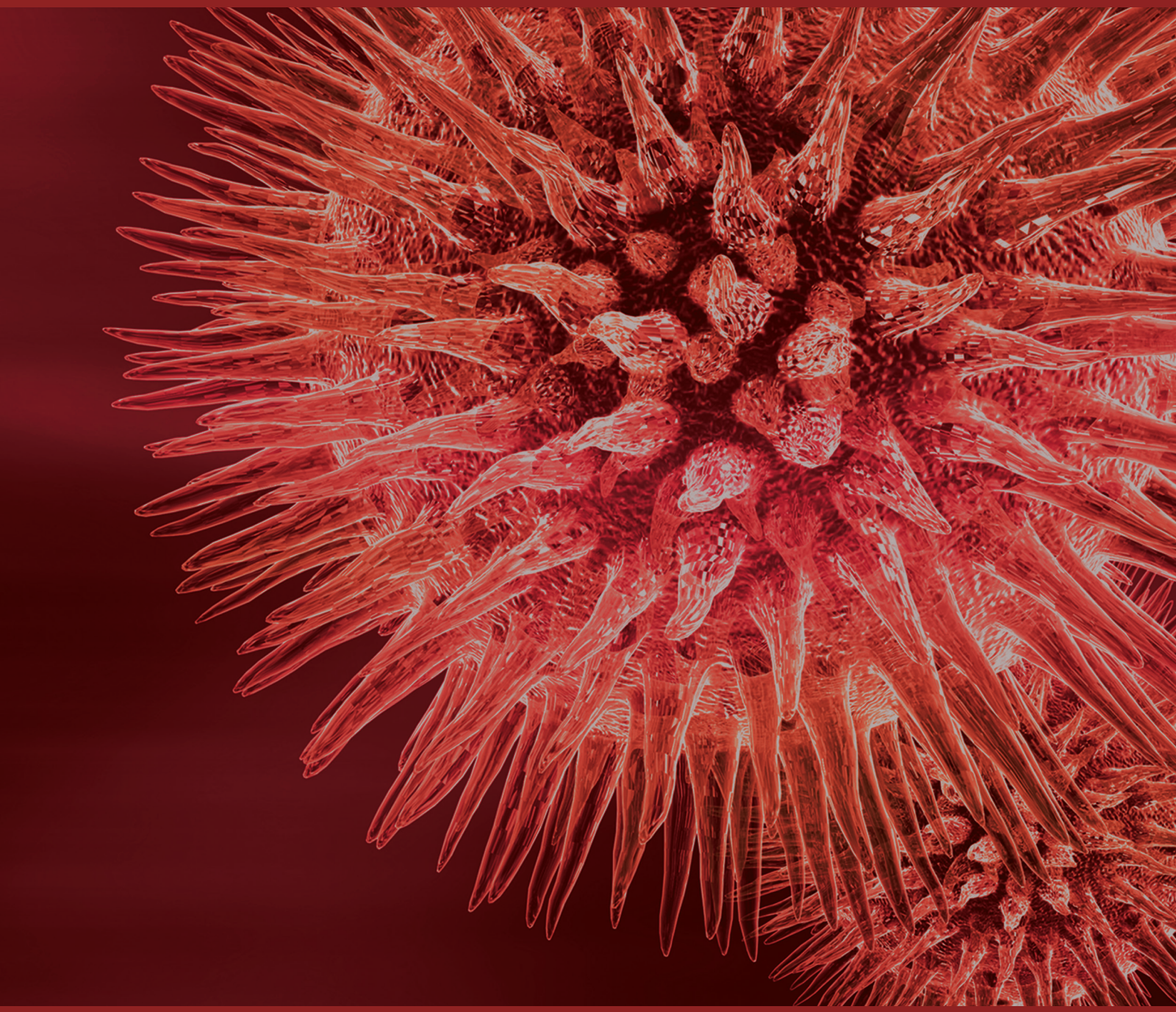
BioMed Research International

Emerging Biomarkers in Renal Damage

Guest Editors: Pasquale Ditonno, Cees van Kooten, Loreto Gesualdo, and Giuseppe Grandaliano



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Editorial

Emerging Biomarkers in Renal Damage

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Acute kidney injury (AKI) is a devastating clinical condition strongly associated with increased morbidity and mortality in critically ill patients. Traditional methods of identifying kidney injury, through measurement of blood urea nitrogen and serum creatinine, are problematic in that they are slow to detect decreases in glomerular filtration rate (GFR) and are influenced by a variety of factors that are not related to GFR changes. Many genes are upregulated in the damaged kidney with the corresponding protein products appearing in plasma and urine. Some of these are candidate markers for more timely diagnosis of AKI. An ideal biomarker of kidney injury would be a substance that the kidney releases immediately in response to damage and that can be detected in the blood or urine without significant metabolism. In recent years, the the introduction of high-throughput omics technologies has led to identification of new biomarkers of renal damage with more favorable test characteristics than creatinine. Advances in this field of research are based on a more detailed understanding of the fundamental biological mechanisms involved in the renal damage progression, as well as on advances in genomic, transcriptomic, proteomic, and metabolomic research.

The purpose of this special issue is to present original research and review articles that provide new insights into molecular pathology underlying the acute and chronic kidney injury and identify novel diagnostic and prognostic biomarkers for these clinical conditions.

Many studies have explored the molecular events associated with the development of tubular atrophy and interstitial fibrosis induced by chronic urinary tract obstruction. Moreover it is well known that the recovery of renal function after relief of ureteral obstruction depends on several factors including the location and duration of the obstruction, whether it is complete or partial, time before relief, and the presence of infection [1]. In their article, G. Lucarelli et al. summarize the role of the emerging urinary biomarkers of obstructive nephropathy based on the current understanding of the pathophysiology of renal injury.

Drug-induced nephrotoxicity plays an important role in the high prevalence and incidence of AKI, especially in neonatal intensive care units. In preterm newborns, one of the most important factors causing the pathogenesis and the progression of AKI is the interaction between the individual genetic code, the environment, the gestational age, and the disease. In this contest, M. Mussap et al. provide a broad overview of the current applications of metabolomics and novel biomarkers for assessing drug-induced toxic nephropathy and AKI in neonatology.

Complement activation is an important mechanism of renal injury in different diseases and in particular in kidney

damage associated with ischemia-reperfusion [2–5]. In this scenario, E. Rodríguez et al. showed that complement pathway was activated in AKI, regardless of the etiology of AKI, leading to the production of lytic complex C5b-C9. Moreover plasmatic membrane attack complex concentrations identified AKI patients at risk of developing serious outcomes like death during hospitalization or unrecovered renal function at time of hospital discharge.

Nowadays the association of a calcineurin inhibitor (CNI) with mycophenolate mofetil (MMF) represents the backbone of solid-organ transplant immunosuppression. Although CNIs (cyclosporine A (CsA) and tacrolimus (FK506)) remain the most effective and widely used immunosuppressive agents in organ transplantation, their prolonged use may result in renal toxicity, renal dysfunction, and irreversible renal failure characterized by extensive tubulointerstitial fibrosis. To minimize the CNIs-associated nephrotoxicity, alternative protocols have been introduced especially with the increasing use of suboptimal donor organs [6–8]. One of these strategies includes the conversion from CsA to other drugs, especially sirolimus (SRL), an inhibitor of the mammalian target of rapamycin (mTOR). Using an experimental model to clarify the pathways of nephropathy evolution in a protocol of conversion from CsA to SRL, J. Sereno et al. demonstrated how conversion to SRL prevented CsA-induced renal damage evolution. Moreover these authors showed that prolonged CsA exposure aggravated renal damage, without clear changes on the traditional markers, but with changes in serums TGF-β and IL-7 and kidney TGF-β and mTOR.

These and other studies published in this special issue underline the need for new diagnostic and prognostic biomarkers. The discovery and validation of biomarkers for AKI will improve the early diagnosis of tubular injury, thereby facilitating timely therapeutic intervention. Novel, sensitive biomarkers should also help to differentiate between etiologies of AKI, predict the severity of renal damage, and provide a tool for differentiating between patients who would benefit from early initiation of renal replacement therapy and those from whom support should be withheld or withdrawn.

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Giuseppe Grandaliano
Giuseppe Lucarelli

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Research Article

Could Pyelonephritic Scarring Be Prevented by Anti-Inflammatory Treatment? An Experimental Model of Acute Pyelonephritis

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Objectives. This study aimed to demonstrate if the addition of anti-inflammatory treatment to antibiotic therapy shows any superiority to the treatment with antibiotic only. *Methods.* Forty-nine Wistar rats were divided into 7 groups. Pyelonephritis was performed by *E. coli* injection to upper pole of kidneys except control group. Group 2 was not treated. Ceftriaxone, ketoprofen, "ceftriaxone + ketoprofen," methylprednisolone, and "ceftriaxone + methylprednisolone" were given in the groups. The technetium-99m-dimercaptosuccinic acid scintigraphies were performed in 3rd day to detect pyelonephritis and 10th week to detect renal scarring. All kidneys were also histopathologically evaluated. *Results.* When 3rd day and 10th week scintigraphies were compared, initial 2.00 ± 0.30 point pyelonephritis score resulted in 0.71 ± 0.36 renal scar score in "ceftriaxone + ketoprofen" group ($P = 0.039$). Initial 2.00 ± 0.43 point pyelonephritis score resulted in 0.86 ± 0.26 renal scar score in "ceftriaxone + methylprednisolone" group ($P = 0.041$). Renal scar score was declined in "ceftriaxone + ketoprofen" group and "ceftriaxone + methylprednisolone" group compared with no-treatment group on 10th week of the study ($P = 0.026$, $P = 0.044$). On histopathological evaluation, it was seen that renal scar prevalence and expansion declined significantly in "ceftriaxone + ketoprofen and ceftriaxone + methylprednisolone" ($P = 0.011$, $P = 0.023$). *Conclusion.* It was evidenced that ceftriaxone treatment in combination with ketoprofen or methylprednisolone declined scar formation in scintigraphic and histopathologic examinations of the kidneys.

1. Introduction

Urinary tract infection (UTI) in infants and children is a relatively common problem, with potentially serious consequences.

Technetium-99m-dimercaptosuccinic acid (DMSA) renal scintigraphy is considered the most sensitive test for the diagnosis of renal involvement and the subsequent development of renal scarring [1, 2].

It may still cause renal scar formation in up to 40% of cases, leading to hypertension, proteinuria, and end-stage renal disease in children [3]. Most important role belongs to acute inflammatory response in scar generation [3, 4].

Various anti-inflammatory treatments were experimental in order to prevent scar generation due to the importance of host origin cytokine in inflammation. Therefore, ongoing research projects are underway to find an agent that can prevent renal scarring and subsequent complications.

Inhibition of acute inflammation in experimental studies by steroids [3, 5], anti-inflammatory agents [6, 7], melatonin [8], pentoxifylline [9], vitamin A [10], vitamins A and E [11], vitamins C and E [12], vitamin E [13], mesenchymal stem cell [14], methylene blue [15], dapsone [16], ulinastatin [17], and montelukast [18] have been reported to reduce kidney damage after infection.

It was thought that both ketoprofen and methylprednisolone may block such mechanisms which give acute inflammatory response, at various stages to prevent renal scar generation. This study aimed to demonstrate if the anti-inflammatory treatment in combination with antibiotic treatment shows any superiority to antibiotic treatment alone.

2. Material and Methods

2.1. Animals. In this study, 49 Wistar rats weighing between 150 and 200 g were used. Animals were housed in specific pathogen-free conditions at room temperature (22°C ± 2°C) using a 12/12-hour light/dark cycle and provided with commercially available rat chow and tap water ad libitum. All of the rats were 8–10 weeks old. Karadeniz Technical University animal ethics board approved number is "02.370."

2.2. Bacteria. The *Escherichia coli* strain UTI 36, isolated from a previous patient with confirmed acute pyelonephritis and phenotyped for the presence of P-fimbria and hemolysin production, was grown overnight on Luria Bertani (LB) agar. Before infection, a single colony of bacteria was inoculated onto LB broth and grown at 37°C with shaking to the stationary phase, after which the organisms were centrifuged and washed twice in phosphate-buffered saline. A solution containing approximately 5×10^9 organisms/mL was prepared. Antibiotic sensitivities were assayed using Kirby-Bauer disks impregnated with ceftriaxone.

2.3. Experimental Infection. All animals were anesthetized by intramuscular injection of ketamine hydrochloride at 80 mg/kg (Ketalar, Parke-Davis). The kidney was exposed through a midline abdominal incision, and 0.1 mL of bacterial solution (5×10^9 colony-forming units/mL) was then injected to upper pole.

2.4. Scintigraphic Imaging. In the 3rd day (48–96th hours) of the study, the technetium-99m-dimercaptosuccinic acid (DMSA) renal scintigraphies of all rats including the control group were taken and the rats were classified according to the presence and expansion of pyelonephritis (Figures 1(a), 1(b), and 1(c)). Furthermore, the DMSA renal scintigraphies of all rats were taken a second time at the 10th week of the study and their kidneys were classified according to the presence and expansion of renal scarring (Figures 1(d), 1(e), and 1(f)).

Scar or pyelonephritis score was assessed using a renal damage score. Each renal unit was divided into three equal zones, and lesions were graded based on percent of affected cortex. Renal scars were each graded by DMSA scan from 0 to 3 according to the extent of pyelonephritic lesions of varying severity involvement as follows: 0, if no damage; 1, if less than 33% damage; 2, if between 33 and 66% damage; 3, if more than 66% damage [5].

2.5. Experimental Groups. The rats were divided equally into seven groups each containing seven rats. In control group, sham operated group (Group 1) consisted of healthy rats. Pyelonephritis was induced by injection of *E. coli* to other rats as mentioned above. In no-treatment group (Group 2), the rats had pyelonephritis but did not receive any treatment. Treatments began 72 hours after bacterial inoculation in the other groups. The rats in ceftriaxone group (Group 3) were treated only with ceftriaxone (i.m) at a dose of 50 mg/kg once daily for 10 days. In ketoprofen group (Group 4), ketoprofen injections were done at a dose of 2 mg/kg for 3 days. Two rats with no indication about infection in 3rd day scintigraphic examination in group 4 were excluded from the study. The rats in "ceftriaxone plus ketoprofen" group (Group 5) were treated with 50 mg/kg ceftriaxone for 10 days and 2 mg/kg ketoprofen for 3 days before 30 minutes of ceftriaxone administration. The rats in methylprednisolone group (Group 6) were given 30 mg/kg methylprednisolone for 3 days. The rats in "ceftriaxone plus methylprednisolone" group (Group 7) were given 50 mg/kg ceftriaxone for 10 days and 30 mg/kg methylprednisolone for 3 days before 30 minutes of ceftriaxone administration.

2.6. Histopathologic Examination. After routine processing, each half renal unit was divided into three equal zones (upper, middle, and lower), and five sections were obtained from anterior zone and five from posterior zone. The sections were obtained through the renal cortex to the collecting system. The sections were stained with hematoxylin-eosin and Masson's trichrome. A pathologist, who was unaware of the group designations, evaluated the specimens. Two main histopathologic changes were regarded as microscopic criteria: the inflammatory response (interstitial mononuclear inflammatory cell infiltration) and cicatrization (interstitial fibrosis-tubular atrophy). These changes were scored semiquantitatively for comparison purposes. The two criteria were each graded from 0 to 3 according to the extent of parenchymal involvement: 0, if none was involved; 1, if less than 5% of the parenchyma was involved; 2, if more than 5% and less than 10% of the parenchyma was involved; and 3, if more than 10% of the parenchyma was involved [8].

2.7. Sacrifice of Animals. The rats in were sacrificed under anesthesia, ten weeks after bacterial inoculation to determine the extent of renal scar formation.

2.8. Statistical Analysis. SPSS (statistical package for social science) for Windows was used for statistical analyses. $P < 0.05$ was regarded as significant. DMSA scintigraphic scores of 3rd day and 10th week are compared with using "Wilcoxon" test. No-treatment group and other groups were compared with "Kruskal Wallis" test on the base of 10th week DMSA scintigraphic results. No-treatment group and other groups were compared with "Mann Whitney U" test on the base of histopathological results.

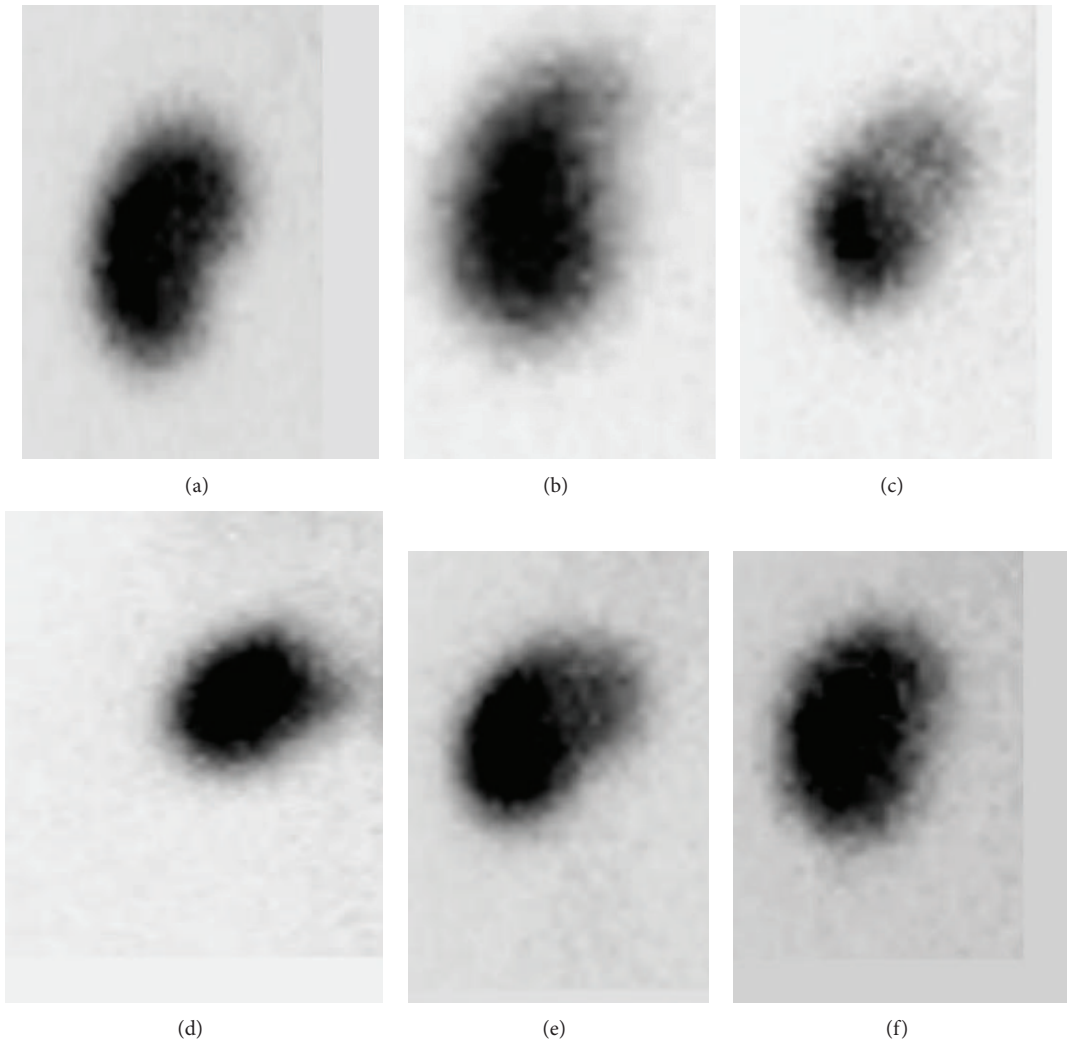

Figure 1: (a) DMSA kidney scintigraphy that indicates normal activity retention in the 3rd day in control group (pyelonephritis score: 0). (b)DMSA kidney scintigraphy in the 3rd day in ceftriaxone group (pyelonephritis score: 1). (c) DMSA kidney scintigraphy in the 3rd day in ceftriaxone-ketoprophen group (pyelonephritis score: 3). (d) DMSA kidney scintigraphy in the 10th week in no-treatment group (scar score: 2). (e) DMSA kidney scintigraphy in the 10th week in ceftriaxone group (scar score: 1). (f) DMSA kidney scintigraphy in the 10th week in Ceftriaxone – Ketoprophen Group (scar score: 0).

3. Results

Table 1 shows the result of DMSA kidney scintigraphy (Figures 1(a)–1(f)) of the 3rd day pyelonephritic scores and of the 10th week renal scarring scores.

When the rats were evaluated in terms of the DMSA renal scintigraphy findings, it was found that in the no-treatment group the pyelonephritic involvement score decreased from 2.00 ± 0.21 in the 3th day to 1.71 ± 0.18 scar score at the end of the 10th week. ($P = 0.157$).

In the group which received only ceftriaxone treatment, the pyelonephritic involvement score was found 1.43 ± 0.29 in 3th day and 1.00 ± 0.30 renal scar score at the end of the 10th week. ($P = 0.083$).

In the group which received "ceftriaxone plus ketoprofen" treatment, the pyelonephritic involvement score decreased from 2.00 ± 0.30 in the 3th day to 0.71 ± 0.36 renal scar score at the end of the 10th week. ($P = 0.039$).

Table 1: Results of DMSA kidney scintigraphy comparison of the groups between themselves with pyelonephritis score in the 3rd day and with scar score of DMSA scintigraphy in the 10th week.

Groups	PN score in 3rd day	Scar score in 10th week	P
Control	0 ± 0	0 ± 0	1.00
No-treatment	2.00 ± 0.21	1.71 ± 0.18	0.157
Ctx	1.43 ± 0.29	1.00 ± 0.30	0.083
Ktp	2.00 ± 0.44	1.20 ± 0.20	0.157
Ctx + Ktp	2.00 ± 0.30	0.71 ± 0.36	**0.039**
Mp	1.71 ± 0.47	1.14 ± 0.26	0.102
Ctx + Mp	2.00 ± 0.43	0.86 ± 0.26	**0.041**

Table 2: Results of DMSA kidney scintigraphy in 10th week no-treatment group and other groups were compared.

Groups	Scar score in 10th week	P
Control	0 ± 0	
No-treatment	1.71 ± 0.18	
Ctx	1.00 ± 0.30	0.080
Ktp	1.20 ± 0.20	0.093
Ctx + Ktp	0.71 ± 0.36	**0.026**
Mp	1.14 ± 0.26	0.100
Ctx + Mp	0.86 ± 0.26	**0.044**

Table 3: Results of histopathological renal scar assessment. No-treatment group and other groups were compared.

Groups		P
Control	0	
No-treatment	1.86 ± 0.40	
Ctx	0.86 ± 0.26	0.053
Ktp	1.20 ± 0.49	0.221
Ctx + Ktp	0.57 ± 0.20	**0.011**
Mp	2.14 ± 0.67	0.946
Ctx + Mp	0.57 ± 0.29	**0.023**

In the group which received "ceftriaxone plus methylprednisolone" treatment, the pyelonephritic involvement score decreased from 2.00 ± 0.43 in the 3rd day to 0.86 ± 0.26 renal scar score at the end of the 10th week ($P = 0.041$).

Table 2 shows no-treatment group compared with other groups on the base of the 10th week DMSA scintigraphic results.

Scar scores were low in the "ceftriaxone plus ketoprofen" and "ceftriaxone plus methylprednisolone" groups when compared with no-treatment group ($P = 0.026$, $P = 0.044$, resp.) as shown in Table 2. Scar score in ceftriaxone treated group was not significant compared to no-treatment group. ($P = 0.080$).

After the histopathological evaluation, when the no-treatment group was compared with other groups in terms of the presence and expansion of renal scars, a statistically significant decrease was observed in the presence and expansion of renal scars in the "ceftriaxone plus ketoprofen" and "ceftriaxone plus methylprednisolone" groups ($P = 0.011$, $P = 0.023$, resp.) (Figures 2(a)–2(d)) (Table 3). Only ceftriaxone treated group is not significantly different compared to no-treatment group ($P = 0.053$).

4. Discussion

Pyelonephritis, an acute infectious disease of kidney parenchyma, now being considered common and serious, bacterial infection that occurs in infancy and early childhood. Renal scarring is a frequent outcome of acute pyelonephritis in children, reported in up to 65% of patients with pyelonephritis. The development of scars in early life, particularly in patients with VUR, has been correlated with the development of hypertension [19], preeclampsia, proteinuria, renal insufficiency, and end-stage renal disease. Of all patients with endstage renal disease, chronic pyelonephritis has been reportedly the cause in 10 to 25% of children, 7–17% in the world, and 23.6% in our country. Antibiotic treatment is important but not minimizing renal damage and scarring alone.

Escherichia coli is the most common organism present up to 80% in UTI as we used in our study, although other enteric organisms such as *Klebsiella* spp. and enterococci, as well as *Staphylococci*, have been identified [20]. Bacterial inoculation in the tissue, ischemia reperfusion damage, and lysosomal lytic enzyme retention cause renal scar by means of endoxines, cytokines, and chimiotaxy [4]. Cytokines play a major role in renal scar formation [3].

It has been reported that renal damage after acute pyelonephritis is more closely related to the extent of the inflammatory process associated with infection than the actual bacterial growth in kidney [21, 22]. The inflammatory response following bacterial inoculation is characterized by recruitment of activated neutrophils and lymphocytes to renal tissue and the release of antibacterial substances such as free radical species and lysosomal enzymes [23]. Therefore, anti-inflammatory treatment is believed to be effective in preventing renal scarring. [5–7, 24, 25]. Glucocorticoids are widely used for the suppression of inflammation [26].

Previous experimental studies reported that technetium-99m-dimercaptosuccinic acid (Tc-99m-DMSA) renal scintigraphy is highly sensitive and reliable for the detection of acute pyelonephritis when performed during the acute phase of infection and renal scaring when performed after recovery. DMSA scintigraphy is considered the most sensitive test for the diagnosis of renal involvement and the subsequent development of renal scarring [1, 2]. The renal cortical changes are acceptably detected by Tc-99m-DMSA renal scintigraphy. In our study, DMSA scan findings were comparable with histopathological results.

Recent experimental studies demonstrate that oxygen-free radical scavengers and antioxidants can reduce tissue damage and renal scaring during acute and chronic pyelonephritis. Antioxidant vitamins [3, 9] increase tissue protection from oxidative stress. Bennett et al. showed that vitamins A and E suppressed renal inflammation in pyelonephritis [11]. Kanter et al. showed that vitamin C treatment alone or with vitamin A may prevent endotoxin-induced renal damage [27]. Imamoğlu et al. measured the level of tissue malondialdehyde in an experimental pyelonephritis model in rats and showed that combined with antibiotics and melatonin it may decrease the inflammation [8]. From the report by Yagmurlu et al., it is showed that anticytokine activity of pentoxifylline could be the other mechanism for the prevention of renal scarring due to pyelonephritis, though this study did not include cytokine measurements [9]. The preventive effect of dapsone which has a scavenging activity on active oxygen species on renal scarring was found to effectively prevent renal scarring by Mochida et al. [16]. Caffeic acid phenethyl ester (an active component of propolis from honeybee hives, which has antioxidant, anti-inflammatory, and antibacterial properties) administration reduced significantly decreased

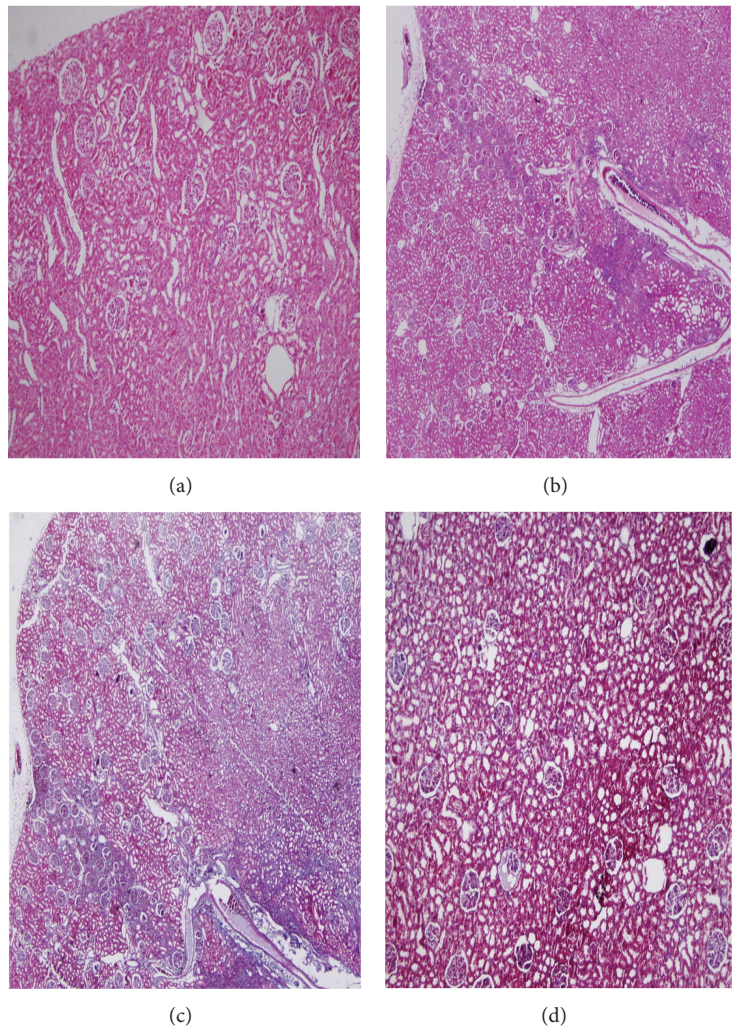

FIGURE 2: (a) Control group kidney tissue which does not indicate any pathological finding in renal parenchyma (H.E ×100). (b) 2nd degree inflammation in renal parenchyma, no-treatment group (HE ×40). (c) 2nd degree scar in renal parenchyma, no-treatment group (M.T ×40). (d) Ceftriaxone-ketoprofen group kidney tissue which does not indicate any pathological findings in renal parenchyma.

E. coli-induced lipid peroxidation as showed by Celik et al. [20]. Cyclophosphamide due to effect of neutropenia and inhibition leukocyte migration of colchicine was used to leukocyte modulation and found that they can prevent renal scarring by Matsumoto et al. But it is not useful because of serious side effects of this agent [28]. In a study by Patel et al. the degree of renal dysfunction and inflammation caused by ischemia-reperfusion was significantly reduced in 5-lipoxygenase knockout mice as compared to wild type mice. Moreover, administration of 5-lipoxygenase inhibitor before ischemia-reperfusion significantly reduced the degree of renal dysfunction and injury [29]. Mesenchymal stem cells (rMSC) were shown to have therapeutic value in alleviating pyelonephritis-associated histopathologic changes in rats [14]. Nevertheless, in real clinical practice, the beginning of the infectious process is silent and cannot be used for antioxidant treatment [13]. Haraoka et al. confirmed the active role of inflammation in renal scarring by demonstrating that prednisolone was sufficient to prevent renal scar formation in rats with APN receiving delayed antibiotics treatment [22]. Surgery performed vezicouretheral reflu on pigs was created as an experimental pyelonephritis model. Pohl et al. investigated the effect of preventing the renal scarring of oral prednisolone [5]. Also, in a study by Sharifian et al. it was concluded that the administration of dexamethasone could possibly prevent the formation of kidney scar [3]. As similar to our study, combined antibiotic with ibuprofen, an inhibitor of cyclooxygenase, and neutrophil chemotaxis was expected to decrease renal scar formation resulting from inflammation by Huang et al. [7].

In this study, it was found that ceftriaxone treatment in combination with ketoprofen or methylprednisolone decreased renal scar development in pyelonephritis. Although there was some decrease in scar expanse compared to the expansion of the pyelonephritic involvement in only ceftriaxone treatment group, this value was not statistically

significant. The rats which received no treatment developed scars whose expansion was proportionate to the expansion of the pyelonephritic involvement.

Compared to previous studies, in all infected rats, pyelonephritis was verified via DMSA and scar verified both DMSA and histopathologic examination. In accordance with the aforementioned studies, our results show that adding ketoprofen or methylprednisolone to ceftriaxone treatment declines scar formation in experimental pyelonephritis. Ceftriaxone treatment is not effective as ceftriaxone plus anti-inflammatory treatment to prevent renal scarring.

Ketoprofen is a nonsteroidal anti-inflammatory drug used for six-month and older infants as analgesic and antipyretic drug approved by Food and Drug Administration and promising good results when used as anti-inflammatory therapy to prevent renal scarring for febrile pyelonephritic children as well as antipyretic effect.

Our study is the first study that showed scar by both DMSA and histopathological examination. The results were consistent with the literature. We hope this information will have potential use in minimizing the renal scarring associated with pyelonephritis in children.

5. Conclusion

Our results provide ceftriaxone with ketoprofen OR methylprednisolone can effectively decrease cellular damage and prevent long-term complications in acute pyelonephritis.

In conclusion, the study showed both histopathologically and with DMSA renal scintigraphy in an experimental pyelonephritis model on rats that the addition of ketoprofen or methylprednisolone to ceftriaxone treatment decreases scar development. The study is the first in the literature in which pyelonephritis was proven by DMSA and pyelonephritic scar was shown both histopathologically and with DMSA. There are few studies on this topic in the literature and our findings are congruent with the literature.

Given the fact that the most common causes of pediatric end stage renal disease in Turkey that develop renal scars are mainly pyelonephritis, the importance of studies and attempts towards the prevention of scars in pyelonephritis is apparent. According to the findings of the study, the addition of ketoprofen or methylprednisolone to ceftriaxone treatment is seen as an intervention that will contribute to the realization of this aim. It is hoped that this study will pioneer the clinical studies to be conducted for the aim of preventing scar development.

Disclosure

Preliminary results of this study are orally presented in the 44th annually scientific meeting of ESPN, Dubrovnik.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Emerging Urinary Markers of Renal Injury in Obstructive Nephropathy

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The effects of obstruction on renal function are the consequence of many factors that profoundly alter all components of glomerular function. Besides the acute effects on glomerular filtration rate and tubule function, a chronic obstruction induces tubular and interstitial injury that results from the activation of different pathways. The progression of tubulointerstitial injury leads to chronic renal damage characterized by tubular atrophy, inflammatory cell infiltration, and interstitial fibrosis. Obstructive nephropathy is an evolving disease in which the renal damage continues even after relief of the obstruction. In particular, it has been demonstrated that the time of relief is the most important factor in predicting long-term renal function deterioration. In this setting, the EGF/MCP-1 ratio, urinary NGAL, and urinary KIM-1 are useful early biomarkers of progressive renal damage and could have a potential role in predicting the long-term renal outcome. This minireview summarizes the role of these emerging urinary biomarkers of obstructive nephropathy based on the current understanding of the pathophysiology of renal injury.

1. Background

The study as well as identification of potential biomarkers for obstructive nephropathy requires an in-depth understanding of the biological pathways involved in the pathogenesis of this disorder. The effects of obstruction on renal function are the consequence of many factors that profoundly alter all components of glomerular function. Besides the acute effects on glomerular filtration rate and tubule function, a chronic unilateral ureteral obstruction (UUO) induces tubular and interstitial injury that results from the activation of different pathways. The progression of tubulointerstitial injury leads to chronic renal damage characterized by tubular atrophy, inflammatory cell infiltration, and interstitial fibrosis [1].

It has been shown that renal tubular injury is the consequence of mechanical stretching, hypoxia, and exposure to oxygen free radicals that result from increased hydrostatic pressure, reduced blood flow, and increased oxidative stress.

In this scenario, three cell types play a fundamental role in the pathogenesis and progression of renal damage: tubular epithelial cells, infiltrating inflammatory cells, and interstitial fibroblasts (Figure 1). During UUO, changes in both the frequency and amplitude of pyeloureteral contractions, in conjunction with sustained urinary pooling proximal to the site of obstruction, contribute to mechanical stretch injury of the tubular epithelium. It has been shown that mechanical stretching of tubular cells is transduced via the extracellular matrix- (ECM-) integrin-cytoskeleton complex [2, 3]. Moreover, recent studies have described the transient receptor potential cationic channel-1 (TRPC-1) as a potential stretch-activated calcium channel expressed in renal cells [4]. Modifications in the intratubular dynamic force as a consequence of obstruction can induce the upregulation and release of transforming growth factor-β (TGF-β) and the activation of tubular apoptosis and the nuclear factor-kB (NF-kB) pathway [2, 5].

In the past year, the deregulation of many cell signaling pathways has been demonstrated in obstructive nephropathy. Among these, the intrarenal renin-angiotensin system plays a fundamental role in orchestrating all the elements that are hallmarks of renal damage. Angiotensin II (ANG II), in particular, can upregulate the expression of many factors such

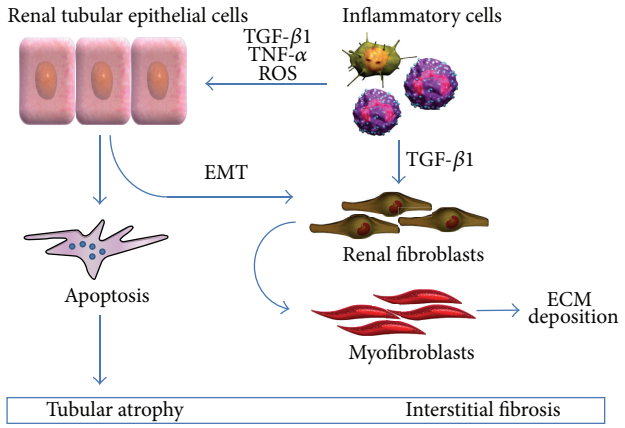

Figure 1: Renal cell types involved in the pathogenesis and progression of obstructive nephropathy. ECM: extracellular matrix; EMT: epithelial to mesenchymal transition; TGF-β1: transforming growth factor-β1; TNF-α: tumor necrosis factor-α; ROS: reactive oxygen species.

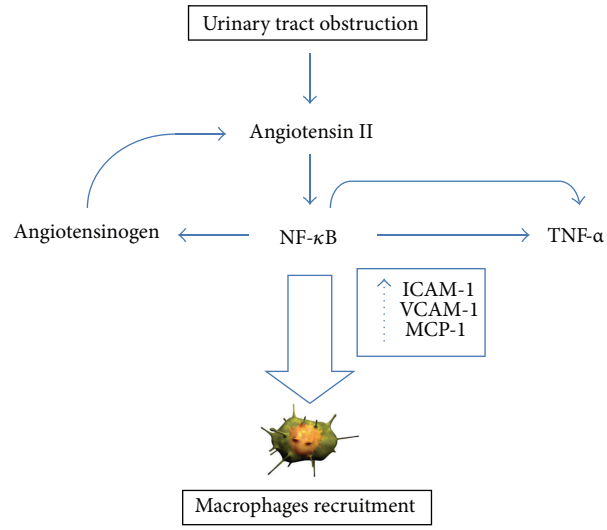

Figure 2: Autocrine-reinforcing loops amplifying angiotensin II (ANG II) and tumor necrosis factor-α (TNF-α) signalling. NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; VCAM-1: vascular cell adhesion molecule-1.

as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α, (TNF-α), osteopontin, vascular cell adhesion molecule-1 (VCAM-1), and NF-kB [6, 7]. ANG II increases the expression of some proliferative factors, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). ANG II also stimulates oxidative stress, through NADPH oxidase activity and the production of reactive oxygen species (ROS) and by increasing NO catabolism. Experimental studies have indicated that protection from obstruction-induced renal damage could be achieved by NO supplementation. This can be accomplished either by inhibiting angiotensin converting enzyme, which increases NO formation, or by stimulating endogenous NO synthase (NOS) [8, 9]. Chronic UUO in mice leads to a significant reduction in inducible NOS (iNOS) activity, and the obstructed kidneys of iNOS knockout mice showed a higher number of apoptotic renal tubules than wild type controls [10].

It has also been demonstrated that the angiotensinogen gene, coding for the precursor of angiotensin, is stimulated by NF-kB activation and an autocrine-reinforcing loop between NF-kB and TNF-α has been described [11, 12]. According to this model, ANG activation II stimulates NF-kB production, which in turn fuels at least two autocrine-reinforcing loops that amplify ANG II and TNF-α formation (Figure 2).

All these factors, along with the upregulation of adhesion molecules such as VCAM-1 and ICAM-1, lead to the recruitment of inflammatory cells within interstitial spaces. These cells, in turn, release additional cytokines with profibrotic and proapoptotic activities, amplifying the tubular damage.

Apoptosis of tubular and interstitial cells is also presumed to be the cause of tubulointerstitial atrophy, secondary to obstructive nephropathy. Apoptosis may be activated by a large number of factors, several of which are present in obstructive nephropathy, such as ischemia, hypoxia, growth factors, cytokines, ANG II, TNF-α, reactive oxygen species, and mechanical stretching. These factors act on a family of cell membrane receptors that include the TNF receptor and Fas. The end result of the activation of these receptors is mitochondrial destabilization and the release of cytochrome C, which subsequently triggers the caspase-mediated apoptotic pathway. The release of cytochrome C is also promoted by the downregulation of antiapoptotic protein bcl-2 [13, 14] (Figure 3).

The activation of all these processes has, as the final common pathway, the development of interstitial fibrosis due to increased deposition of ECM, cell infiltration, tubular apoptosis, and induction of the epithelial to mesenchymal transition (EMT). This latter process involves the tubular cells and is characterized by the downregulation of epithelial markers (such as E-cadherin, ZO-1, and cytokeratins), upregulation of mesenchymal proteins (including vimentin, α-smooth muscle actin, and FSP-1), loss of cell adhesion molecules, invasion of basement membrane, and migration in the interstitium, where these cells acquire myofibroblast characteristics [15]. In this compartment myofibroblasts induce collagen accumulation and are the main ECM-producing cells. Renal fibrosis is also induced by different cytokines and growth factors. Among these, TGF-β1 is the most powerful profibrogenic factor involved in kidney diseases and the major mediator of renal injury during UUO.

In this minireview we summarize the role of the emerging urinary biomarkers of obstructive nephropathy based on the current understanding of the pathophysiology of renal injury.

2. Urinary Epidermal Growth Factor (EGF), Monocyte Chemotactic Protein-1 (MCP-1), and EGF/MCP-1 Ratio

Many studies have explored the molecular events associated with the development of tubular atrophy and interstitial fibrosis induced by chronic urinary tract obstruction. Notably,

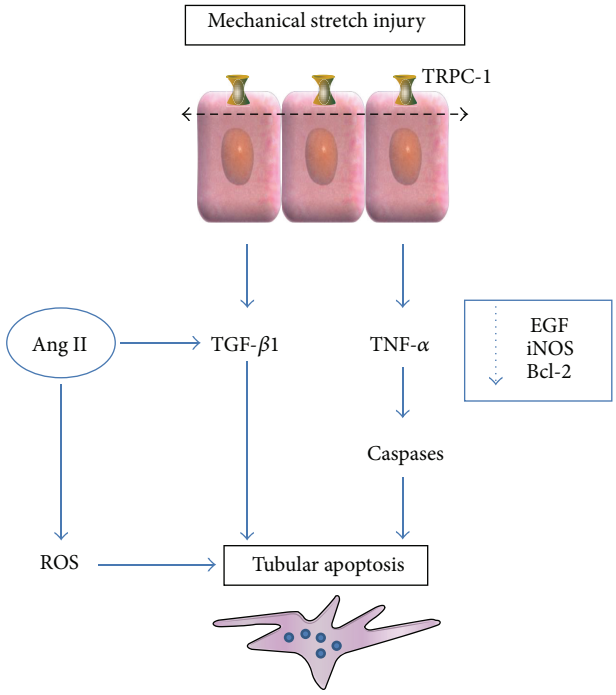

Figure 3: Pathogenesis of renal tubular apoptosis in obstructive nephropathy. Ang II: angiotensin II; EGF: epidermal growth factor; iNOS: inducible NO synthase; ROS: reactive oxygen species; TRPC-1: transient receptor potential cationic channel-1.

ureteral obstruction determines a significant increase in monocyte chemotactic protein-1 (MCP-1) expression and a decrease in epidermal growth factor (EGF) expression by tubular cells [7–10]. MCP-1 is a specific chemokine that promotes monocyte chemotaxis, and its expression at the tubular level drives the recruitment of these inflammatory cells within the interstitial space of the obstructed kidney. An increased expression of this protein has been observed in different tubulointerstitial diseases [16–19].

MCP-1 renal expression and urine excretion are strictly related to tubular damage and the extent of monocyte infiltration. As described above, in an obstructed kidney, tubular epithelial cells release a number of autocrine factors and cytokines, including ANG II, TGF-β1, and TNF-α [20–24]. These factors, along with overexpression of adhesion molecules, lead to infiltration of the renal interstitium by inflammatory cells, including macrophages. These, in turn, release additional cytokines.

All of these factors accelerate the development of interstitial fibrosis by increasing the extracellular matrix, epithelial to mesenchymal transition, cell infiltration, and tubular apoptosis. On the other hand, EGF, a polypeptide produced by the ascending portion of Henle's loop and by the distal convoluted tubule, modulates tubular cell growth and tissue response to injury in the kidney with tubulointerstitial damage [25–28]. In obstructive nephropathy a downregulation of EGF, Bcl-2, and antioxidant enzymes has been observed in association with an increased production of superoxide and hydrogen peroxide, contributing to an increased rate of apoptosis and tubular dropout. EGF administration reduces UUO-induced renal damage by increasing tubular proliferation and reducing apoptosis, tubular atrophy, and interstitial fibrosis. In view of these findings, a reduced urinary EGF/MCP-1 ratio has been proposed as a marker of acute and chronic damage in human renal diseases [16, 17, 29].

The deregulation of these molecular pathways is associated with tubulointerstitial fibrosis and permanent loss of renal function, which may continue to progress even after the obstruction has been relieved. In a neonatal rat model, Chevalier et al. showed the progression of renal interstitial collagen accumulation after relief of UUO [30]. Moreover, in another study, the same authors demonstrated that despite relief of five-day obstruction in the neonatal period, the growth of the kidney in adulthood was impaired. In particular a significant reduction of nephron number in the postobstructed kidney was observed, in association with an increased expression of interstitial α-smooth muscle actin and macrophage infiltration [31]. Ito et al. demonstrated, in adult rats, that even if the renal blood flow and GFR of an obstructed kidney returned to control levels after relief of a short-term ureteral obstruction, in the long term renal function was compromised by progressive interstitial fibrosis and tubular atrophy [32]. It is well known that the recovery of renal function after relief of ureteral obstruction depends on several factors including the location and duration of the obstruction, whether it is complete or partial, and the presence of infection. In particular, time before relief seems to be the most critical issue. A recent study showed that patients who underwent delayed relief of a ureteral obstruction had a decreased long-term renal function and were at risk for arterial hypertension [33]. Moreover, the median urinary EGF/MCP1 ratio was significantly higher in the subgroup of patients who underwent repair of the ureteral lesion within 2 weeks compared to those with a later repair. A direct correlation was found between MAG3 clearance of the obstructed unit and the EGF/MCP-1 ratio, as well as an inverse correlation between the urinary cytokines ratio and time before repair. The role of the EGF/MCP-1 ratio as a marker of renal damage has also been explored in children with congenital obstructive nephropathy. Grandaliano et al. observed a significant reduction of EGF urinary excretion in subjects affected by congenital ureteropelvic junction obstruction (UPJO) compared to healthy children, in association with a marked increase of MCP-1 levels [16]. Moreover, the MCP-1 urine concentration was significantly higher in patients with recurrent urinary infections. These findings are in agreement with other studies showing the same pattern of gene expression at renal tubulointerstitial level for these molecular markers [34–36]. A recent study confirmed the diagnostic role of the urinary EGF/MCP-1 ratio in a paediatric population affected by UPJO and suggested that these biomarkers may help to follow the progression of parenchymal damage in obstructed renal units [17].

3. Neutrophil Gelatinase-Associated Lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kDa protein of the lipocalin family [37]. It is not an

organ-specific protein and is secreted by different tissues, including the respiratory, gastrointestinal, and urinary tracts. NGAL overexpression has been described in different pathological conditions such as inflammation, sepsis, ischemia, renal damage, and cancer. In the kidney, this protein is synthesized in the thick ascending limb of Henle's loop and collecting ducts [38, 39]. The expression of this protein is rapidly induced in response to renal tubular injury, and increased levels of serum and urinary NGAL (uNGAL) have been reported in the setting of different renal diseases such as acute kidney injury (AKI), diabetic nephropathy, nephritic syndrome, tubulointerstitial damage, and IgA nephropathy [40–43].

The induction of NGAL serves to limit tubular injury, even apart from its bacteriostatic properties. Although NGAL is synthesized in the distal nephron, it has been suggested that NGAL could act on the proximal nephron through its uptake from the circulation by tubular epithelia via endocytosis. According to this two-compartment model, in the setting of sepsis or renal disease, urinary NGAL is produced by local distal tubule synthesis, whereas proximal tubule NGAL derives from the circulating pool [39].

The role of NGAL as a biomarker of AKI was recently reviewed by Haase-Fielitz et al. [44]. In their meta-analysis, the authors found that serum or uNGAL levels represented a valuable early predictor of renal damage and that high concentrations had a prognostic role in predicting the progression to renal replacement therapy and mortality. A recent case-control prospective study evaluated the role of urinary NGAL in a population of children with severe hydronephrosis caused by UPJO [45]. The findings of this study showed that the uNGAL/creatinine ratio was significantly higher in obstructed patients compared to normal subjects. Moreover, three months after surgery, uNGAL values had decreased and did not significantly differ from the control group. Similar findings have been confirmed by more recent studies. In particular, Cost et al. showed, in a cohort of children with UPJO, that renal pelvis uNGAL levels were higher than bladder uNGAL and that these values were significantly higher compared to the levels measured in a control population of unaffected children [46]. Moreover, the renal pelvic uNGAL levels were inversely correlated with the function of the affected renal unit. The authors concluded by suggesting the potential usefulness of this biomarker for selecting patients at risk for renal function deterioration and candidates for reconstructive surgery.

4. Kidney Injury Molecule-1 (KIM-1)

Kidney injury molecule-1 (KIM-1) is a member of the type I transmembrane glycoprotein structurally characterized by an N-terminal region containing an IgV-like and a mucin domain. In humans, KIM-1 is undetectable in healthy subjects but is strongly expressed and released by injured proximal tubular epithelial cells. These characteristics, along with the persistent expression in tubular cells until damage recovery, and the rapid cleavage of its ectodomain which can be detected in urine contribute to making KIM-1 an ideal biomarker of tubular injury [47].

Clinical studies have shown that urinary KIM-1 (uKIM-1) is higher in patients with ischemic renal injury compared to controls and also that it is a predictor for the risk of developing AKI [48–50]. Additional studies have documented that KIM-1 and its urinary derivative are upregulated in various kidney diseases including diabetic nephropathy, focal glomerulosclerosis, membranoproliferative glomerulonephritis, and IgA nephropathy [51]. Urinary KIM-1 also predicts graft loss in kidney transplant recipients [52–54] and this predictive role has an important role in the era of kidney transplants from expanded criteria donors [55, 56].

In recent years there has been a growing interest in tumor markers not only for diagnostic purposes but also to improve the predictive power of clinical and pathological factors in prognostic models [57–59]. It has been shown that KIM-1, besides its utility as a biomarker of renal injury, could have a diagnostic role in renal cell carcinoma (RCC).

RCC accounts for about 3% of all adult malignancies and, even if many proteins have been investigated as potential biomarkers [60] in recent years, their diagnostic and prognostic relevance is still under debate. In this scenario, Han et al. have demonstrated the expression of KIM-1 in RCC tissue samples. Moreover, uKIM-1 was detectable in the urine of RCC patients before nephrectomy but showed markedly reduced levels after surgery [61].

Considering the characteristics of uKIM-1, which contribute to its utility as an early and sensitive biomarker for kidney injury, its role was recently analyzed in the setting of obstructive nephropathy. In a case-control prospective study performed in children with severe hydronephrosis due to UPJO, Wasilewska et al. showed that, like uNGAL, uKIM-1 concentrations were significantly higher in affected children compared to control groups [45]. Moreover, three months after surgery, uKIM-1 values had decreased significantly even if they were still higher than the concentrations found in children with dilated not obstructed kidneys. A more recent study investigated the diagnostic performance of uKIM-1 and uNGAL for AKI in 90 patients with obstructive nephropathy [62]. Both uKIM-1 and uNGAL concentrations were higher in AKI patients than non-AKI patients, and the uKIM-1 value measured 72 hours after surgery was an independent predictor of renal outcome in patients with AKI.

5. Conclusions

In the last decades much has been learned about the pathophysiology of obstructive nephropathy. This better knowledge has led to the discovery of novel biomarkers for diagnostic and prognostic purposes. We have learned that obstructive nephropathy is an evolving disease in which the renal damage continues even after relief of the obstruction. In particular, it has been demonstrated that the time of relief is the most important factor in predicting long-term renal function deterioration. Patients who underwent late surgical relief of an obstruction suffer from decreased renal function in the long term, as a consequence of the molecular events triggered by previous acute injury, which lead to progressive interstitial fibrosis and tubular apoptosis.

In this setting, the EGF/MCP-1 ratio, uNGAL, and uKIM-1 are useful early biomarkers of progressive renal damage and could have a potential role in predicting the long-term renal outcome. Additional comprehensive validation studies are warranted to confirm the utility of these biomarkers in clinical practice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Giuseppe Lucarelli and Vito Mancini contributed equally to this work.

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Review Article

Emerging Biomarkers and Metabolomics for Assessing Toxic Nephropathy and Acute Kidney Injury (AKI) in Neonatology

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Identification of novel drug-induced toxic nephropathy and acute kidney injury (AKI) biomarkers has been designated as a top priority by the American Society of Nephrology. Increasing knowledge in the science of biology and medicine is leading to the discovery of still more new biomarkers and of their roles in molecular pathways triggered by physiological and pathological conditions. Concomitantly, the development of the so-called "omics" allows the progressive clinical utilization of a multitude of information, from those related to the human genome (genomics) and proteome (proteomics), including the emerging epigenomics, to those related to metabolites (metabolomics). In preterm newborns, one of the most important factors causing the pathogenesis and the progression of AKI is the interaction between the individual genetic code, the environment, the gestational age, and the disease. By analyzing a small urine sample, metabolomics allows to identify instantly any change in phenotype, including changes due to genetic modifications. The role of liquid chromatography-mass spectrometry (LC-MS), proton nuclear magnetic resonance (^{1}H NMR), and other emerging technologies is strategic, contributing basically to the sudden development of new biochemical and molecular tests. Urine neutrophil gelatinase-associated lipocalin (uNGAL) and kidney injury molecule-1 (KIM-1) are closely correlated with the severity of kidney injury, representing noninvasive sensitive surrogate biomarkers for diagnosing, monitoring, and quantifying kidney damage. To become routine tests, uNGAL and KIM-1 should be carefully tested in multicenter clinical trials and should be measured in biological fluids by robust, standardized analytical methods.

1. Introduction

In neonatology, the evaluation and the monitoring of kidney function continues to be a complex, intriguing, and interesting medical investigation involving the close cooperation of several specialists belonging to pediatric critical care, neonatology, neonatal nephrology, obstetrics, radiology, and laboratory medicine. Drug-induced nephrotoxicity plays an important role in the high prevalence and incidence of neonatal acute kidney injury (AKI), which in turn is the most important cause of morbidity and mortality in preterm babies admitted to neonatal intensive care units (NICUs) [1], especially in those conditions characterized by the absence of oliguria [2]. In fact, the immature preterm kidney with ongoing nephrogenesis is likely to be vulnerable to the hemodynamic changes associated with preterm birth. The early stages of toxic nephropathy and AKI are commonly characterized by very few, nonspecific clinical signs and by nonsignificant variations of conventional serum and urine biomarkers. During toxic nephropathy, the renal functional reserve may mask parenchymal lesions, as estimated by urinalysis, glomerular filtration rate (GFR), blood urea nitrogen (BUN), and serum creatinine (SCr), up to the point where over 75% of the functioning nephrons have been lost [3, 4]. Accordingly, these factors measure incipient kidney failure and in most cases, the finding of normal results does not mean the absence of kidney dysfunction. These drawbacks call for new methods, namely, biomarkers that can identify early and accurately

kidney damage and impairment, avoiding the risk of neonatal death and of complications in childhood and adulthood.

2. Next Generation Biomarkers for Toxic Nephropathy and AKI

Identification of novel drug-induced toxic nephropathy and AKI biomarkers has been designated as a top priority by the American Society of Nephrology. The concept of developing a new toolbox for earlier diagnosis of disease states is also prominently featured in the National Institute of Health (NIH) Road Map for biomedical research. In 2007, the Acute Kidney Injury Network (AKIN), a collaborative group of investigators from all major critical care and nephrology societies, proposed a staging system based on 3 categories (mild, moderate, and severe) in a way similar to those (risk, injury, and failure) used by the RIFLE staging system. In children, A modified pediatric RIFLE (pRIFLE) classification was proposed in which similar criteria were used for pediatrics [5]. Despite these working classification systems, the diagnosis of AKI is problematic, as current diagnoses rely on two functional abnormalities: functional changes in serum creatinine and oliguria. Both of these are late consequences of injury and not markers of the injury itself. The increasing application in clinical practice of the so-called "omics," especially metabolomics, seems to offer new attractive perspectives for improving neonatal outcome and management in kidney disease and, more extensively, in critically ill newborns (Figure 1).

3. Kidney Development in the Perinatal Period

Human kidney development involves two basic processes: morphologic formation and, ultimately, the acquisition of function. The first one occurs exclusively in utero from the 6th to the 36th week of gestation, whereas the second one starts during the fetal life and accelerates after birth to reach adult levels. In preterm newborns, postnatal renal development exhibits accelerated maturation with a reduced width of the nephrogenic zone, reduced percentage of immature V-stage glomeruli, and increased number of glomerular generations [6]. Immaturity worsens the natural neonatal kidney vulnerability to ischemic and hypoxic insults, mainly caused by higher perfusion rate, and vulnerability to potentially endogenous- or exogenous-toxic substances that may be present in the circulation (drugs, bilirubin, etc.) [7]. Immaturity of renal tubular cells might involve the expression of transporting molecules, the regulation of transporting systems, and the way by which different tubule segments interact. The physiological renal immaturity cannot be considered a risk factor for healthy full-term infants fed an appropriate diet; however, it becomes a major risk in extremely low and very low birth weight (ELBW and VLBW, resp.) preterm infants, often affected by various systemic diseases (dehydration, congestive heart failure, systemic inflammation and sepsis, abrupt changes in intrarenal hemodynamics, etc.) and by inappropriate losses, mechanical ventilation, and exogenous pharmacologic stress. Multiple factors may play a role in the epigenetic modulation of kidney development, including maternal diet, stress and hypertension, drugs administered to the mother or to the newborn, prematurity, low birth weight (LBW), and intrauterine growth retardation (IUGR) [8, 9]. All these factors may lead to a disturbance of nephrogenesis, resulting in low nephron numbers at birth, which may represent the main factor favoring the development of hypertension and, eventually, of end stage renal disease (ESRD) in childhood or adulthood [10]. Finally, the considerable interindividual variability in kidney maturation, recently confirmed by autopsy studies in preterm infants, represent a major risk factor of progressive renal disease in adulthood [11, 12].

4. Conventional Biomarkers of Drug-Induced Toxic Nephropathy and Acute Kidney Injury

The current diagnosis of drug-induced nephrotoxicity and AKI relies on a marker of steady-state kidney function, muscle-derived SCr. Unfortunately, neonatal age is typically marked by a non-steady-state condition and even AKI itself represents a very unstable pathological condition. Therefore, SCr becomes a retrospective, insensitive, and even deceptive measure of kidney injury [13–15]: retrospective because SCr concentration may result in a very delayed signal even after considerable kidney injury, it must accumulate over many days, a length of time that is regulated by extrarenal modifiers such as muscle mass and diet [16]; insensitive because as much as a 50% loss of renal function may be required to elevate SCr enough that it comes to medical attention, whereas levels that fall short of this threshold are usually dismissed, despite their known association with excess mortality and prolonged hospitalization, and as SCr is affected by tubular secretion and systemic production, changes in SCr concentration are not specific to tubular injury; deceptive because SCr level often reflects transient physiologic adaptations to volume changes or the presence of chronic kidney disease (CKD), rather than AKI. Most importantly, the measurement of SCr does not identify the cell type that is acutely injured, even though this localization determines the natural history of the disease and its response to therapy. Because small changes in SCr are associated with short- and long-term adverse events, as demonstrated previously [17], determining whether the increase in SCr represents structural damage or a reversible functional change takes on some urgency, as therapeutic strategies are somewhat different [18]. BUN is also widely used for evaluating kidney function; however, likewise SCr, BUN is not a reliable surrogate biomarker of kidney injury because various factors may affect its concentration. For example, an increase in BUN concentration can be found with volume depletion in the absence of any tubular injury. Furthermore, BUN increases concomitantly with the increase of urea synthesis, as occurs with endogenous (catabolic states or blood in gastrointestinal tract) or exogenous (protein supplementation) protein loads [19].

5. The Omics Era and Its Impact on the Study of Neonatal Kidney Diseases

With the latest advances in high-throughput technologies, the pace of advances in the "omics" fields that are relevant

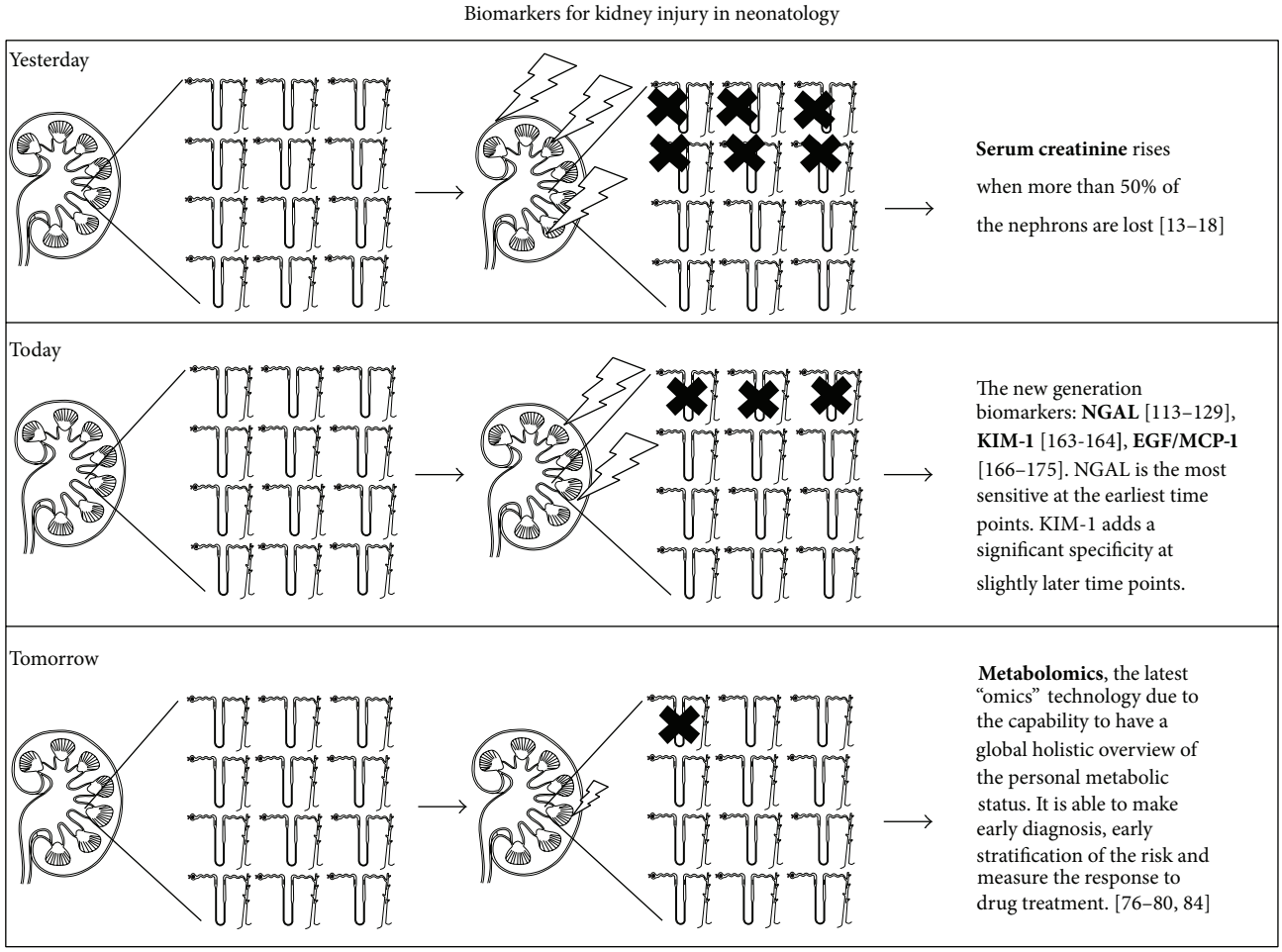

Figure 1: Schematic progresses in knowledge on biomarkers for kidney disease and damage. Abbreviations: NGAL = neutrophil gelatinase-associated lipocalin; KIM-1 = kidney injury molecule-1; EGF/MCP-1 = epidermal growth factor/monocyte chemotactic peptide-1 ratio.

to clinical medicine has markedly accelerated [20]. The widespread availability of enabling technologies such as functional genomics and proteomics has accelerated the rate of novel biomarker discovery and therapeutic targets for kidney diseases [21]. For example, great attention has been focused on the study of genetic changes contributing to specific renal pathology that could lead to CKD, such as IgA nephropathy and idiopathic membranous nephropathy [22]. Genomics, proteomics, and metabolomics, when taken together as a whole, provide a comprehensive framework, also referred to as systems biology that describes the biochemical function of an organism and its response to challenges. The advent of the microarray, or cDNA chip, allows investigators to search through thousands of genes simultaneously, making the process very efficient. Such gene expression profiling studies have identified several genes whose protein products have emerged as CKD and AKI biomarkers [23, 24]. However, known gene polymorphisms explain only a fraction of associated risk, suggesting that sequence variations in the human genome are only part of the puzzle leading to the evolution of the nascent field of epigenetics [25]. A large number of epidemiology studies suggest that the environment is a major factor in disease etiology [26, 27]. Epigenetics refers to heritable modifications in gene function without alteration of DNA sequences [28]; concisely, epigenetics changes regulate gene expression [29]. The best-known examples of epigenetics modification are DNA methylation and chromatin remodeling by modification of histone proteins [30]; these modifications are potentially reversible and are not associated with changes in DNA sequence [31]; furthermore, they specify functional outputs from the DNA template and are often heritable through cell division [32]. The unifying theme of epigenetic disease is a disruption of normal phenotypic plasticity [33]. Epigenetics alterations are involved in the pathogenesis and progression of kidney disease, especially because these alterations are easily promoted by the plethora of coexisting metabolic alterations and inflammation associated with CKD [34]. Recent reports of epigenetics mechanisms in renal injury, fibrosis, inflammation, and metabolic memory have set the stage for future research in this area [35]. Advancing technologies have radically improved the speed and precision of identifying and measuring proteins in biological fluids, and proteomic approaches are also beginning to yield novel biomarkers for assessing kidney damage [36].

Proteomics can be operationally defined as a field of study that is focused on the identification of proteins, peptides, or their interactions and posttranslational modifications [37]. Clinical proteomics is currently conducted to detect or select biomarkers of disease; mass spectrometry (MS) is the central analytic technique used for most investigational proteomics [38]. In the early 2000's it was introduced as the concept of protein profiling: the fusion of MS technique with pattern recognition, where specific peak profiles, without knowledge of individual peak identity, were treated as biomarkers [39]. In particular, matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) and surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) MS can profile proteins of low molecular weight (LMW) as well as the metabolic products of serum proteins, originating the so-called peptidome [40]. Briefly, proteinases generate biomarker fragments and circulating protein fragments generated in the diseased tissue microenvironment may serve as diagnostic protein markers. Research studies on the peptidome revealed an apparent abundance of LMW proteins and peptides that potentially contain disease-specific information and showed that changes in the expression patterns of these molecules may be disease specific. Peptidome is a promising high-throughput approach for identifying new potential biomarkers in various body fluids; in particular, urinary peptidome profiling with high-throughput methods such as MB-MALDI-TOF MS or SELDI-TOF MS appears to be a promising tool in nephrology research [41]. Several research papers have demonstrated that "urinary peptidome" may be a resource at least as dynamic and informative as the "urinary proteome" [42, 43].

6. Metabolomics for Managing Neonatal Kidney Disease

Genomics, transcriptomics, and proteomics identify genotype and phenotype. On one hand, the genotype of a patient defines the risk or probability of reacting to a disease, drug, or environmental challenge in a certain way; genotype can be considered "static." On the other hand, the phenotype more closely reflects clinical reality at any given moment, and it may be considered "dynamic." The advent of metabolomics, in which all of the metabolites in a given tissue or biological fluid are examined (with the caveat that some metabolites will not be detected in any given experiment), is one of the latest advances in the field of omics. The mRNA over- or underexpression (identified as transcriptome) translates directly into corresponding up- or downregulated expression of proteins (surrogate biomarkers), respectively. However, changes in the transcriptome are not necessarily associated with changes in signal transduction and cell biochemistry; therefore, downstream confirmation by analyzing protein concentrations and/or metabolites is commonly performed [44]. In turn, variations of protein levels in biological fluids, cells, and tissues may also not necessarily translate into changes in cell biochemistry and function, since protein expression is not always correlated with activity. Main causes include reaction with oxygen radicals, changes in translational modifications, and allosteric regulation by substrates, products, and other inhibitors and activators. Metabolomics offers several advantages over genomics, transcriptomics, and proteomics, making it extremely attractive for research and clinical purposes. Firstly, metabolites vary both quantitatively and qualitatively at any given time, and this is of great interest, because in most cases pathophysiological pathways and histological damages are directly caused by cell metabolism. Secondly, while transcriptomics and proteomics may be considered "late signals" since their response to a challenge may take hours, days, and sometimes weeks, metabolic response, on the other hand, can be measured very often within seconds or minutes. Thirdly, transcriptomics and proteomics strictly detect endogenous changes, whereas the metabolome communicates with the environment and is an open system. Last but not least, despite the very high overall number of endogenous metabolites (~100,000), the number of major metabolites relevant for clinical diagnostics and drug development has been estimated at 1,400–3,000 molecules [45], which means less data to manipulate and interpret, being genes (~25,000), transcripts (~85,000), and proteins (>10,000,000) greatly outnumbered. It is reasonable to argue that metabolomics is typically more closely associated with a disease process or drug effect than proteins, mRNA, or genes [46].

At first, metabolomics was defined as "the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [47]. More recently, the same authors have revised the definition of metabolomics as "a global holistic overview of the personal metabolic status," or in other word, "a snapshot of the chemical fingerprints that specific cellular processes leave behind" [48]. The metabolome was first defined as "the quantitative complement of all of the LMW molecules present in cells in a particular physiological or developmental state" [49]; more concisely, the metabolome can be considered the phenotype reflecting the epigenetics modifications [50]. Two strategies configure metabolomics studies: the targeted and the nontargeted approach [51]. The latter may be defined as a "nonspecific approach," investigating all the metabolites (both endogenous and exogenous) detectable in a fluid or tissue; this analysis is focused on capturing as much information as possible, providing a functional fingerprint of the physiological and pathological state of the body. The former is focused on the investigation of several well-defined compounds (e.g., those discovered in a new metabolic pathway); it is only used when the target of a drug or disease process is at least partially understood. Metabolic fingerprinting describes the unbiased analysis of the metabolome by examination of metabolite patterns in different experimental groups with the subsequent classification of these patterns into a fingerprint [52]. Samples can be classified if the metabolite fingerprints differ between groups allowing for sample clustering. Metabolite identification relies on public databases [53]: the human metabolome Data Base (HMDB) is the metabolomic equivalent of GenBank. It is an open access database (http://www.hmdb.ca/) providing reference to nuclear magnetic resonance (NMR) and mass spectra, metabolite disease associations, metabolic pathway

data, and reference to metabolite concentrations for hundreds of human metabolites from several biofluids [54, 55].

In most cases, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and MS based assays are used for metabolic fingerprinting [56–59]; these techniques require a well-defined sample preparation [60]. Typically, ^{1}H NMR spectroscopy allows for the simultaneous detection of 20–50 metabolites with an analytical sensitivity ranging 1–10 μmol/L [61]; below this cutoff, the detection and quantification of metabolites is still unreliable, although high field NMR spectroscopy and cryoprobes can improve sensitivity [62]. On the other hand, MS is still considered the gold standard in metabolite detection and quantification; depending on the metabolite, the sensitivity of MS is in the picomolar and nanomolar range. However, MS should be coupled to an array of separation techniques including gas chromatography (GC) and liquid chromatography (LC); in addition, MS requires longer analytical time (20–60 min for each sample), extensive sample preparation including derivatization and the limitation to volatile compounds [63]. Other technologies less commonly used for metabolomics are Raman and infrared spectroscopy [64, 65]. Each method has serious drawbacks, such that neither by itself is ideal.

In general, biological fluids are considered highly adequate for metabolomics, because they closely represent quantitative and qualitative variations of phenotypic molecular markers such as metabolites. In neonatal and pediatric nephrology, however, urine is considered the ideal sample, since it is a so-called "proximal matrix," being closer to (or in direct contact with) the kidney, which is the site of disease or drug effect under investigation [66]. This means that urine metabolome better reflects kidney pathophysiological changes, while metabolome in whole blood, plasma, and serum better reflects systemic changes. Furthermore, urine represents an "open system" by which the body through the elimination of water, ions, metabolic degradation, and harmful or toxic substances regulates important balance, maintaining homeostasis. It is also of importance that the urine metabolome includes the intermediate metabolites, which reflects specific metabolic processes. Finally, urine can be collected easily (a spot sample is adequate) and noninvasively: these aspects are of extreme importance in neonatology, especially for preterm babies LBW. Two conditions are essential to perform metabolomics studies on urine samples: first, urine must be collected in a sterile bag or plastic container, because bacteria metabolism significantly interferes on the urine metabolome. Secondly, urine samples must be frozen at −80°C immediately after collection, until analysis [67].

Metabolomics allows to: (a) identify unknown molecular mechanisms; (b) select molecular markers that can be used for drug discovery, preclinical, and clinical drug development; (c) develop diagnostic tools. Theoretically, metabolomics has a great potential in nephrology for identifying metabolic patterns as markers of kidney function, disease, and injury and for elucidating and monitoring pharmacodynamic and toxicodynamic molecular mechanisms [68]. Interestingly, ^{1}H NMR-based metabolomics permits to follow metabolism in different areas of the kidney, which could yield important information about nephrotoxicity [69]. Monitoring renal transplantation and allograft rejection are also promising applications for metabolomics [70, 71]. In the neonate, the continuous, abrupt changes in renal hemodynamics, fluid balance, glomerular and tubular functions, and metabolism due to the developmental transition from fetal to neonatal life make it critical for the analysis of the metabolic profile and the research of new molecules associated with pathological conditions. In particular, the body water content significantly differs between premature babies (85%), infants (75%), and adults (50–60%). In addition, the amount of water in the extracellular compartment is almost double in the newborn compared with that in the adult (40% versus 20%, resp.) [72]. However, metabolomics is opening up new perspectives to improve the management of sick newborns and of VLBW and LBW preterm newborns by providing new metabolic profiles and biomarkers associated with perinatal/neonatal maturational processes and their metabolic background [73–75]. In particular, different urine metabolic profiles were found between 26 full term and 41 preterm babies [76]. Interestingly, the urine metabolome discriminated preterm babies with a GA between 23 and 32 weeks from those with a GA between 33 and 36 weeks. Single metabolites recognizing unambiguously these groups were: hippurate, tryptophan, phenylalanine, malate, tyrosine, hydroxybutyrate, N-acetylglutamate, and proline. Furthermore, metabolomics seems to be a valuable tool for investigating the pharmacokinetics and the effectiveness of drugs in neonatology [77, 78]. In a clinical study in 21 children with nephrouropathies compared with 19 healthy controls, it was found that renal and urinary tract malformations are associated with specific urine metabolic profiles never overlapping at least in part urine metabolic profile in healthy controls [79]. Metabolomics may play a key role in perinatology, particularly for searching biomarkers of IUGR. In a group of preterm babies with IUGR diagnosed by ultrasonography during pregnancy, urine metabolic profile revealed an increase in the flux of the urea cycle, amino acid metabolism, glycine, serine, and threonine metabolism [80]; interestingly, it appeared to be associated with a significant increase of myoinositol levels in comparison to the control group ($P = 0.04$). Although the role of myoinositol is still unclear, it may be associated with the development of metabolic syndrome. The metabolic profiles in broncoalveolar lavage fluid (BALF) were recently investigated in 12 preterm babies with respiratory distress syndrome (RDS) during mechanical ventilation and at extubation time point, after surfactant administration [81]. By using the GC-MS technical approach, 25 overexpressed metabolites were identified, including 10 with known molecular structure. Metabolomics has been successfully used for managing pediatric asthma, pneumonia, and bronchiolitis [82]. Metabolomics seems to have the capacity to assess the risk of CKD in adulthood in subjects born with ELBW. By comparing the urine metabolic profile in 19 healthy young adults (mean age 24 y) born with ELBW with that of 13 healthy adults of similar age (controls) born at term appropriate for gestational age (AGA), we found two totally

distinct cluster regions of metabolites: the first one associated with controls and the other one with subjects born ELBW [83]. By multivariate analysis, the most important discriminating metabolites between the two groups were N-methylhydantoin, glycine, valine, and glutamine. Metabolites significantly increased in ELBW urine samples were correlated to CKD as well as to the metabolic syndrome. Several studies have attempted to find early diagnostic surrogate biomarkers for a variety of renal diseases as well as to direct personalized therapies; in particular, metabolomics has been applied to the study of uremic syndrome, diabetic nephropathy, AKI, polycystic kidney disease, and kidney cancer [84].

Although individual data sets including genomic, epigenomic, proteomic, and metabolomic information are highly informative, integrating them together offers the exciting potential to answer many long-standing questions. From this point of view, metabolomics should be considered complementary to transcriptomics and proteomics. Therefore, integrative analysis has become an essential part of experimental design in the era of next-generation genomics and is no longer the domain of bioinformatics technicians [85].

7. Neutrophil Gelatinase-Associated Lipocalin (NGAL)

On the basis of experimental studies on kidney injury in mouse and other animal models, researchers picked the 10 proteins that were most overexpressed in the kidney for further study. Of those, neutrophil gelatinase-associated lipocalin (NGAL) turned out to be a useful marker. NGAL has emerged as the most promising marker of AKI in a number of clearly defined clinical contexts [86]. Human NGAL also named human neutrophil lipocalin (HNL), lipocalin-2 (*lcn2*) or lipocalin 24p3, siderocalin, α_1-microglobulin-related protein, and uterocalin is a ubiquitous 25-kDa glycoprotein consisting of 178 amino acid residues belonging to the lipocalins family [87]. NGAL binds and transports LMW proteins (ligands) as well as lipophilic substances, including the bacterial siderophore enterochelin from gram-negative bacteria, bacilli bactin from gram positive, and carboxymycobactins from Mycobacteria. When these siderophores are bound to NGAL, iron transfer to bacteria is prevented and growth is blocked [88]. In the course of experimental studies inducing kidney ischemia-reperfusion, it was observed that NGAL has the capacity to attenuate the extent and the severity of renal tissue injury by reducing apoptosis and enhancing proliferation of renal tubules [89]. This effect is due to the iron delivery to proximal tubular cells by NGAL; iron, in turn upregulates heme oxygenase-1, a well-known enzyme that protects tubular cells [90]. NGAL can additionally promote renal tubular formation and might enhance tubule repair after AKI [91].

NGAL was firstly isolated from the supernatant of human activated neutrophils [92]; later, it was evident that infection and inflammation, oxidative stress, cytokines, ischemia, cancer, intoxication, and other conditions leading to cellular necrosis, apoptosis, and death induce the rapid upregulation of NGAL synthesis in epithelial cells of various human tissues, including liver, lung, kidney, and trachea [93, 94]. NGAL is thought to be an acute-phase protein with upregulated expression in different inflammatory conditions as well as in cancer [95]; it has also been suggested that NGAL comprises a critical component of innate immunity to exogenous bacterial infections [96]. In healthy subjects, circulating NGAL is filtered through the glomerulus and is then captured by megalin within the proximal tubule, where it traffics to lysosomes and degrades to a 14-kDa fragment being not recycled [97, 98]. Experimental studies on animal models have definitively demonstrated that the response of the kidney to injury consists of the NGAL mRNA overexpression by distal tubular cells and collecting ducts [99]; similarly, the pivotal role of NGAL in regulating the progression of CKD to AKI was demonstrated [100]. A growing body of evidence indicates that NGAL increases within a few minutes in both serum and urine after an injury of kidney tissue (up to 1,000-fold) and thus it has been widely evaluated in clinical studies for the early diagnosis, monitoring, and risk stratification of AKI and other kidney diseases.

AKI induces a rapid and massive upregulation of NGAL mRNA within the thick ascending limb of Henle's loop and in the collecting ducts, originating the so-called "NGAL renal pool" [101]; the accumulation of NGAL in the distal nephron leads to a significant increase in urine NGAL (uNGAL), which represents the major fraction of kidney tissue-derived NGAL. Simultaneously, AKI induces NGAL mRNA upregulation in the liver, in the lung, and in various distant organs, originating a rapid release of NGAL into the circulation, called "NGAL systemic pool." Finally, uNGAL may originate both from circulating NGAL and from the distal nephron, and this hypothesis has been recently reported as "two-compartment model of NGAL trafficking during AKI" [102]. In this model, systemic NGAL that is produced in the setting of sepsis or renal disease may serve to limit proximal tubular damage, whereas NGAL synthesized locally in the kidney may exert bacteriostatic effects in the distal urogenital tract. According to this model, changes in uNGAL concentration may better predict AKI than those in plasma, being earlier and more specific.

With the intent to introduce the determination of NGAL in clinical practice, new analytical methods have been developed and optimized in biological fluids; in particular, NGAL can be measured in urine by a reliable and automated method, easily adaptable in an emergency setting [103]. Being NGAL a critical component of innate immunity to bacterial infection, it is also expressed during systemic inflammation and sepsis, and thus it increases significantly in the bloodstream and, in turn, in urine. Moreover, during systemic inflammation and sepsis uNGAL significantly increases because of neutrophils accumulation within the tubular lumen. Consequently, uNGAL can increase (a) as a result of a renal tubular damage; (b) in the course of an acute phase response; (c) as the concomitant presence of sepsis with AKI. How can we distinguish sepsis-induced uNGAL from AKI-induced uNGAL excretion? Three isoforms of human NGAL have been isolated: a 25-kDa monomer, a 45-kDa disulfide-linked homodimer, and a 135-kDa heterodimer consisting of a monomer covalently bound with neutrophil

gelatinase, also named matrix metalloproteinase (MMP-9) via an intermolecular disulfide bridge. The NGAL/MMP-9 complex formation seems to protect MMP-9 enzymatic activity from degradation [104]. Neutrophils synthesize the monomer and the homodimer, whereas renal tubular epithelial cells synthesize the monomer and, to some extent, the heterodimer [105, 106]. Therefore, we can speculate that an "ideal" immunoassay capable to distinguish various molecular forms of uNGAL should permit to assess the origin of uNGAL and, ultimately, the pathological process leading to the changes in uNGAL concentration [107]. Unfortunately, this "ideal" immunoassay does not exist; more important, developing AKI in the course of sepsis and developing sepsis in the course of AKI are both dynamic pathological processes in a continuous interaction [108].

NGAL has emerged as a very promising biomarker of kidney injury and damage especially because kidney epithelia express and excrete massive quantities of NGAL within 30 minutes into urine when stressed by ischemia-reperfusion injury, nephrotoxins, sepsis, and chronic progressive changes [109, 110]. These findings have been confirmed in various studies in adults, children, and newborns [111, 112]. A milestone in clinical studies evaluating NGAL as a biomarker for AKI is that of Mishra et al., published in 2005 [113]. In a group of 71 children undergoing cardiothoracic surgery, which represents an excellent model of renal ischemia-reperfusion, the development of AKI in 28% of children was detected by substantial increase in serum and urine NGAL 2 hours after cardiac surgery. Importantly, NGAL detected AKI 34 hours earlier than serum creatinine did. Both urine and plasma NGAL were powerful independent predictors of AKI, with an AUC of 0.998 for the 2-hour urine NGAL and 0.91 for the 2-hour plasma NGAL measurement. A conspicuous number of studies on NGAL for assessing AKI in the course of cardiac surgery have subsequently confirmed the results published by Mishra [114–120]. In a prospective study, uNGAL was measured immediately after kidney transplantation and then for subsequent 3 times every 6 hours [121]. NGAL urine levels significantly differed between patients with delayed graft recovery, patients with slow graft function, and patients with immediate graft function; results clearly showed that uNGAL can be used as an early, noninvasive and accurate predictor of the need for dialysis within the first week of kidney transplantation, confirming a previous similar study performed mainly on children [122]. Urine NGAL has also been shown to predict the severity of AKI and dialysis requirement in a multicenter study of children with diarrhea-associated hemolytic uremic syndrome [123]. The measurement of plasma and urine NGAL seems to be a reliable, predictive biomarker of AKI following contrast administration and in the intensive care setting [124, 125]. In the neonate, uNGAL is detectable at birth, showing a wide range of variability in premature newborns (0.51–2815.7 μg/L), probably because NGAL plays an important developmental role in proliferating nephrons of premature kidneys [126]. Urine NGAL was found to be inversely related to birth weight [126]. The sensitivity of uNGAL in detecting oliguria (used as a surrogate of AKI) was found low (31%) while specificity was 90%, suggesting that babies who do not have clinical indicators such as oliguria would test negative for AKI when using uNGAL as a screening mechanism. Reference ranges for uNGAL were established in 50 VLBW premature babies (2–150 μg/L) by an immunoblot assay employing human NGAL recombinant to create the standard curve [127]. Finally, uNGAL can be considered an early biomarker of sepsis in VLBW newborns, discriminating babies with late onset blood culture positive sepsis from those with single blood culture positive for *S. epidermidis* and from those with negative blood culture treated with antibiotics [128]. Despite the fact that NGAL is emerging as a center-stage player in the AKI field as a novel predictive biomarker, large multicenter studies to further define the predictive role of plasma and urine NGAL as a member of the putative "AKI panel" have been initiated [129].

8. Kidney Injury Molecule-1 (KIM-1)

Kidney injury molecule-1 (KIM-1) is a biomarker for renal proximal tubular damage discovered only about 15 years ago [130]. KIM-1 is a type I cell membrane glycoprotein containing, in its extracellular portion, a six-cysteine immunoglobulin-like domain, two *N*-glycosylation sites, and a Thr/Ser-Pro rich domain characteristic of mucin-like *O*-glycosylated proteins [131]. The cytoplasmic domain of KIM-1 is relatively short and possesses a potential phosphorylation site, indicating that KIM-1 may be a signaling molecule; the ectodomain is cleaved by metalloproteinases. KIM-1 is also known as T cell immunoglobulin mucin domains-1 (TIM-1), as it is expressed at low levels by subpopulations of activated T cell [132]; another KIM-1 homolog is an African green monkey protein cloned as hepatitis A virus cellular receptor-1 (HAVCR-1), expressed by hepatocytes [133]. The KIM-1 gene is markedly upregulated in the postischemic rat kidney; a large pharmaceutical company consortium, using an unbiased genomic approach to evaluate genes upregulated with the nephrotoxin cisplatin, determined that KIM-1 was upregulated more than any of the 30 000 genes tested [134].

KIM-1 is a phosphatidylserine receptor on renal epithelial cells that recognizes and phagocytizes apoptotic cells commonly present in the postischemic kidney; this function has the property to transform normal proximal tubule cells into a phagocyte [135]. As a result, KIM-1 is involved in the clearance of the apoptotic debris from the tubular lumen and thus may play an important role in limiting the autoimmune response to injury since phagocytosis of apoptotic bodies is one mechanism for limiting the proinflammatory response [136]. KIM-1 positive atrophic tubules are usually surrounded by fibrosis and inflammation; this association suggests that KIM-1 might be involved in the development of interstitial fibrosis [137]. In normal human and rodent kidney, mRNA and protein are expressed at very low levels [138]; when an injury (e.g., hypoxia and ischemia) affects the kidney, mRNA KIM-1 levels increases more than any other known transcript and the protein is localized at very high levels on the apical membrane of proximal tubule in that region where the tubule is most affected. The cell surface (mature) form of KIM-1 is a 104 kDa peptide. After injury the ectodomain of KIM-1, consisting of a 90 kDa soluble protein (soluble KIM-1), is shed from proximal tubular kidney epithelial cells into urine

[139, 140]. Soluble KIM-1 may form a protective layer on the proximal tubular cells, thereby protecting them from protein casts forming within the lumen. *In situ* hybridization and immunohistochemistry revealed that KIM-1 is expressed in dedifferentiated proximal tubular epithelial cells in damaged regions, especially in the S3 segment of the proximal tubule in the outer strip of the outer medulla, a region that is highly susceptible to injury as a result of ischemia or toxins. Because KIM-1 colocalizes with markers of proliferation, it was suggested that KIM-1 plays a role in the regeneration process.

A large number of studies in animal models have provided robust evidences that KIM-1 is expressed in the affected segments of the proximal tubule whenever a toxin or pathophysiological state results in dedifferentiation of the epithelium [141]. Dedifferentiation is a very early manifestation of the epithelial cell response to injury [142]. In particular, KIM-1 induction has been demonstrated after ischemic renal tubular injury and necrosis [143]. KIM-1 is also expressed in other conditions where proximal tubules are dedifferentiated, including toxic nephropathy from cyclosporine [144], cadmium [145], and other toxic compounds [146], and in renal cell carcinoma [147, 148]. In a protein-overload model of tubulointerstitial disease, KIM-1 was found markedly induced within tubular cells and conspicuously excreted into urine [149], suggesting that it is involved in the pathogenesis of proteinuria-induced renal damage/repair and that its urine levels may serve as a marker of proteinuria-induced renal damage. In mice, KIM-1 was found upregulated in polycystic kidney disease especially in regions of the kidney where fibrosis takes place [150]. Extrarenal functions for KIM-1 have been described in the immune system, where the mouse *kim-1* gene is a susceptibility locus for experimental allergic asthma [151], and human KIM-1 (TIM-1) is involved in the regulation of T_H2 cytokine production. The molecular mechanisms regulating KIM-1 urinary levels have been recently elucidated [152]. KIM-1 shedding can be enhanced dramatically by pervanadate, a potent inhibitor of protein tyrosine phosphatases. The constitutive and pervanadate-induced shedding of KIM-1 is mediated by metalloproteinases and regulated by extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), respectively. The protein secondary structure in the juxtamembrane region of KIM-1 is important for its cleavage. Ectodomain cleavage of KIM-1 results in generation of a truncated 14-kDa cell membrane-associated and tyrosine-phosphorylated KIM-1 fragment [152].

KIM-1 is a specific histological biomarker for diagnosing early tubular injury in renal biopsies: it was found that positive staining in proximal tubules correlates very well with renal dysfunction, being a very useful biomarker to diagnose kidney epithelial cell injury in renal allografts [153]. When a renal transplant recipient has renal dysfunction without acute cellular rejection detectable in the renal biopsy, negative staining of KIM-1 suggests that renal dysfunction is not associated with tubular injury and may be attributed to prerenal factors. Clinical studies have reported that urinary excretion of KIM-1 is an independent predictor of long-term graft loss and therefore a promising new biomarker in early prediction of graft loss [154, 155]. KIM-1 can be considered an outstanding biomarker for kidney injury for at least three reasons: first, it is not detectable in normal kidney, second it is expressed by the affected segment of the proximal tubule whenever the initial ischemic or toxic insult induces dedifferentiation of the epithelium, and third the ectodomain of KIM-1 is shed from injured cells, being excreted into urine within 12 h and persisting over time before regeneration of the epithelium [156]. Urinary KIM-1 concentration is closely correlated with the severity of kidney injury, representing a noninvasive and sensitive surrogate biomarker for diagnosing, monitoring, and quantifying kidney damage [157]. Due to these superb characteristics, the Predictive Safety Testing Consortium (PSTC), a cooperation group consisting of members from the biotech and pharmaceutical industry together with members from academia, from US Food and Drug Administration (FDA), and from the European Medicines Agency (EMA), has included KIM-1 in the short list of biomarkers under investigation to detect drug-induced nephrotoxicity [158, 159]. KIM-1 ectodomain soluble protein can be measured by a microsphere-based Luminex xMAP technology employing polyclonal antibodies raised against the human KIM-1 ectodomain; this method requires only few microliters of urine sample. The lower limit of detection for this assay is 4 ng/L, and the inter- and intraassay variability, expressed as coefficient of variation (CV, %), is less than 10%. In healthy subjects, urine KIM-1 excretion expressed as mean ± standard deviation (SD) is 58 ± 8.0 ng/day, whereas in untreated patients with nondiabetic proteinuria KIM-1 excretion is 1706 ± 498 ng/day [160]. This microbead technique is an adaptation of the previously described sandwich ELISA assay, which is known to mirror findings by western blot analyses [161, 162].

Very few studies investigated the clinical usefulness of KIM-1 in newborns, infants, and children. In a case-control prospective study performed in 20 children aged 0.16–17 years with severe congenital hydronephrosis (HN) caused by ureteropelvic junction obstruction (UPJO), the urine KIM-1 levels were significantly elevated in subjects developing an obstructed kidney but not yet undergone pyeloplasty [163]. Three months after surgery, the concentration of urine KIM-1 decreased significantly but did not reach the values found in a group of 20 children with dilated but not obstructed kidney (mild nonobstructive HN). Urine levels of KIM-1 were negatively correlated with differential renal function (DRF) assessed by the radionuclide scan. The strong negative correlation between KIM-1 level in the pelvic urine and DRF of the affected kidney confirms that urine KIM-1 ectodomain soluble protein is closely related to tissue KIM-1 and with the severity of renal damage. Recently, urinary KIM-1 was measured in a cohort of 123 premature newborns in a NICU: in 52 babies with GA ≤26 weeks, KIM-1 expressed as geometric mean and 95% confidence intervals, was 226 ng/L (184–277 ng/L). As GA increased, KIM-1 progressively declined, being geometric mean 158, 155, and 143 ng/L in babies with GA ranging between 26–28, 28–30, and 30–36 weeks, respectively [164]. An advantage of KIM-1 over uNGAL is that it appears to be more specific to ischemic or nephrotoxic AKI and is not significantly affected by prerenal azotemia, urinary tract infections, or CKD.

9. Opportunity and Challenges for Utilizing Monocyte Chemoattractant Protein-1 and Epidermal Growth Factor as Biomarkers of Kidney Damage and Repair

Progressive CKD involves the impairment of several tracts of the nephron by the activation of pathological processes, specifically glomerulosclerosis, tubulointerstitial fibrosis, and vascular sclerosis. Of these, tubulointerstitial changes are greatly relevant in determining the progression of kidney damage; indeed, the severity of tubular atrophy, interstitial cell infiltration, and fibrosis correlates with the decline of kidney function. Most of cell infiltrates are monocytes and differentiated interstitial macrophages: they play a central role in innate immune protection both early, by a cytotoxic and proinflammatory action, and later, by phagocytizing cellular debris and apoptotic bodies in order to initiate the process of tissue repair [165]. Concomitantly, monocytes and differentiated interstitial macrophages generate radical oxygen species, nitric oxide, complement factors, and proinflammatory cytokines leading to a direct damage to resident cells. Over the past 15 years, several studies both on animal model and on patients with kidney disease have reported encouraging results on the clinical utility of biomarkers overexpressed by renal tubular cells and by monocytes-macrophages infiltrating the peritubular space. In patients with chronic tubulointerstitial injury, the urinary excretion rate of C-C motif chemokine ligand 2 (CCL2), also called monocyte chemoattractant protein-1 (MCP-1) and that of epidermal growth factor (EGF) together with the calculation of the ratio EGF/MCP-1 seem to represent powerful prognostic indexes, opening new perspectives for the early, accurate evaluation of tubulointerstitial injury and repair. In addition, MCP-1 gene activation in patients with kidney injury is reflected by increased urinary excretion of MCP-1 and thus it may be a useful biomarker of AKI, since it mediates acute ischemic and toxic kidney injury, as demonstrated elsewhere [166]. The severity of progressive interstitial fibrosis is strongly correlated with the extent of macrophage infiltration in the peritubular space, which in turn is positively correlated with the expression of chemokines (chemotactic cytokines constituting a large family of peptides classified into four subfamilies). MCP-1 belongs to the CC chemokine subfamily (β-chemokine); it is a potent chemotactic factor for monocytes and macrophages. MCP-1 gene is located on chromosome 17 (17q11.2-q21.1) and the mature form of the protein is composed of 76 amino acid residues with a molecular weight of 13 kDa. The major source of MCP-1 is monocytes and macrophages. In biopsy specimens from patients with acute interstitial nephritis, MCP-1 was found clearly upregulated; the gene and the protein expression were primarily localized in tubular and glomerular parietal epithelial cells, as well as in infiltrating monocytes and macrophages [167]. In addition, in patients with immunoglobulin A nephropathy, urinary excretion of MCP-1 was higher than that in healthy subjects and positively correlated with the renal MCP-1 gene expression [167]. On the other hand, human EGF is a 6 kDa peptide consisting of 53 amino acid residues synthesized by the ascending portion of the Henle's loop and the distal convolute tubule. Human EGF is a peptide growth factor inducing epithelial cell growth and metabolism; various experimental and clinical studies have found that EGF acts as a mediator of normal tubulogenesis and tubular regeneration after injury [168, 169]. In a rat model involving neonatal and adult rats with chronic unilateral ureteral obstruction, prolonged administration of EGF attenuated the impairment of renal development in the maturing rat kidney affected by chronic unilateral ureteral increased the proliferation of renal tubular epithelial cells obstruction and suppressed apoptosis [170]. Progressive increase over time in urinary EGF excretion has been demonstrated in asphyxiated babies put on assisted ventilation [171] as well as in the course of therapeutic treatment of children with recurrent urinary tract infection and in those with vesicoureteric reflux [172]. The calculation of the EGF/MCP-1 ratio has been proposed as a better index of the relationship between renal tubular regeneration and interstitial inflammation; as previously demonstrated, an inverse relationship exists between renal gene expression of EGF and MCP-1 [173]. In a cohort of 132 patients with biopsy-proven IgA nephropathy, the urinary EGF/MCP-1 ratio showed a better ability to predict outcomes rather than the two single measures, leading to the conclusion that it may be considered a prognostic index of ESRD: at the cutoff value of 23.2, sensitivity was 88.9% and specificity 86.4% [174]. EGF/MCP-1 ratio was found significantly downregulated in two groups of untreated children with ureteropelvic junction obstruction compared with controls; in addition, surgical treatment of urinary obstruction improved significantly EGF/MCP-1 ratio when compared with the group of obstructive ureteropelvic junction obstruction [175]. On the basis of the current available results from the literature, it is desirable to perform further multicenter trials in order to validate definitively these biomarkers, taking into account the importance to early assess the capacity of repair of the renal tubular cells.

10. Conclusions

Recent progress in medical care has contributed to improved survival among all but the most immature infants [176]. In LBW and VLBW the mortality rate continues to be high and AKI plays an important role in reducing survival in these babies [177]. Conventional biomarkers of toxic nephropathy and AKI, such as oliguria, SCr, and BUN, are insensitive and cannot be considered markers of injury. Unfortunately, the management of critically ill newborns is often crucial for the absence of specific symptoms and signs related to kidney impairment and damage. Increasing knowledge in the science of biology and medicine has accelerated the discovery of novel biomarkers and elucidated their roles in molecular pathways triggered by physiological and/or pathological conditions. Emerging tools, like metabolomics, depend on sophisticated technologies (LC-MS, GC-MS, ^{1}H NMR, etc.) which play a pivotal role, contributing to the sudden development of new biochemical and molecular tests. There is an urgent need to translate these developing methods (epigenomics, metabolomics, etc.) and next generation biomarkers (NGAL, KIM-1, MCP-1, etc.) from bench to bedside in order

to improve clinical outcome and quality of care in acute ill newborns and infants. Metabolomics seems to be a very promising tool minimizing false positive and false negative results. Interestingly, metabolomics may become a powerful tool for reducing health care costs associated with length of hospitalization, appropriateness in drug administration, severe complications, hospital-acquired infections, and so forth. It is likely that NGAL and KIM-1 will emerge as tandem biomarkers of AKI, with NGAL being most sensitive at the earliest time points and KIM-1 adding significant specificity at slightly later time points. This combination may be an excellent opportunity to improve the efficacy of the therapeutic treatment in sick newborns and, in turn to reduce the risk of complications that may significantly affect the quality of life in childhood and adulthood. Metabolomics together with epigenetics and proteomics is leading to the transformation of conventional medicine in personalized medicine, integrating multiple levels of information; they represent a challenge for promoting, maintaining and improving the health of populations through translational research.

List of Non-Standard Abbreviations (Alphabetic Order)

AKI:	Acute kidney injury
^{1}H NMR:	Proton nuclear magnetic resonance
AGA:	Appropriate for gestational age
AKIN:	Acute kidney injury network
BALF:	Broncoalveolar lavage fluid
BUN:	Blood urea nitrogen
CKD:	Chronic kidney disease
CV:	Coefficient of variation
DRF:	Differential renal function
EGF:	Epidermal growth factor
EMA:	European medicines agency
ERK:	Extracellular signal-regulated kinase
ESRD:	End stage renal disease
ELBW:	Extremely low birth weight
FDA:	Food and drug administration
GC:	Gas chromatography
GFR:	Glomerular filtration rate
HAVCR-1:	Hepatitis A virus cellular receptor-1
HMDB:	Human metabolome data base
HN:	Hydronephrosis
HNL:	Human neutrophil lipocalin
IUGR:	Intrauterine growth retardation
KIM-1:	Kidney injury molecule-1
LBW:	Low birth weight
LC:	Liquid chromatography
Lcn-2:	Lipocalin-2
LMW:	Low molecular weight
MALDI-TOF:	Matrix-assisted laser desorption and ionization time-of-flight
MAPK:	Mitogen-activated protein kinase
MCP-1:	Monocyte chemotactic peptide-1 ratio
MMP-9:	Matrix metalloproteinase-9
MS:	Mass spectrometry
NGAL:	Neutrophil gelatinase-associated lipocalin
NICU:	Neonatal intensive care unit
NIH:	National Institute of Health
pRIFLE:	Pediatric risk, injury, failure, end stage renal disease
PSTC:	Predictive safety testing consortium
RDS:	Respiratory distress syndrome
SCr:	Serum creatinine
SD:	Standard deviation
SELDI-TOF:	Surface-enhanced laser desorption and ionization time-of-flight
TIM-1:	T cell immunoglobulin mucin domains-1
uNGAL:	Urine NGAL
UPJO:	Ureteropelvic junction obstruction
VLBW:	Very low birth weight.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Value of Plasmatic Membrane Attack Complex as a Marker of Severity in Acute Kidney Injury

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The aim of this study was to determine if complement pathway is activated in AKI; for this purpose, we measured, through ELISA sandwich, the terminal lytic fraction of the complement system, called membrane attack complex (C5b-C9), in AKI patients compared with patients with similar clinical conditions but normal renal function. Our data showed that complement system is activated in AKI. Plasmatic MAC concentrations were significantly higher in AKI patients than in those with normal renal function; this difference is maintained independently of the AKI etiology and is proportional to the severity of AKI, measured by ADQI classification. In addition, we found that plasmatic MAC concentrations were significantly higher in patients who did not recover renal function at time of hospitalization discharge, in patients who died during the acute process, and in patients who need renal replacement therapy during hospitalization, but in this last group, the differences did not reach statistical significance. In conclusion, plasmatic MAC concentration seems valuable as a marker of AKI severity.

1. Introduction

Epidemiological studies have reported that the risk of adverse outcomes is proportional to the severity of acute kidney injury (AKI) [1–4]. Accurate identification of patients with severe renal injury early in the disease course could augment the efficacy of available interventions and improve patient outcomes. However, it is difficult to estimate the severity of AKI at an early time point because AKI staging is based on the magnitude of changes in serum creatinine and urine output, surrogates of glomerular filtration rate (GFR) that do not change until renal injury has occurred [5–7]. The recent Kidney Disease Improving Global Outcomes clinical practice guideline for AKI (K-DIGO) highlighted the need for improved risk assessment for patients with established AKI [8].

Many biomarkers have been proposed as early markers of AKI, which may be useful for the detection of AKI before increases in serum creatinine, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), IL-18, cystatin C, liver-type fatty acid-binding protein, monocyte chemoattractant protein 1 (MCP-1), prepro-epidermal growth factor (EGF), and urinary components of renin-angiotensin system [9–16]. These biomarkers seem to show different aspects of renal injury; cystatin C concentrations correlate with changes in glomerular filtration rate whereas NGAL concentrations are related to tubular stress or injury [17–20]; urinary EGF excretion was reduced in cisplatin nephrotoxicity, in ischemic kidney injury [21], and after ureteral obstruction supressing tubular apoptosis and enhancing renal tubular cell regeneration [22, 23]. Munshi et al. showed that urinary MCP-1 may be a useful biomarker of AKI, providing the first evidence that urinary histone assessment may be a useful tool in kidney disease [24].

These biomarkers change with treatment or recovery, which suggests that they may be used to monitor interventions [25].

Novel biomarkers increase our understanding of the pathogenesis of AKI by identifying possible mechanisms of injury. Currently NGAL is the most studied renal biomarker

and probably the most promising of them because of the results obtained in different scenarios and clinical conditions [26–30].

Complement activation is an important mechanism of renal injury in different diseases affecting each of the renal compartments (glomerulus, tubulointerstitium, and vascular departments) [31]. The complement system is an important innate humoral defense system comprised of more than 20 plasma proteins that may be activated in a cascade fashion by either the classic pathway (immune complex mediated) or the alternative pathway. A regulatory system of both plasma proteins and membrane bound proteins acts to prevent the inappropriate activation of complement by autologous cells [31].

Complement activation has been shown to be an important event in the development of ischemic AKI in mice. Studies in complement-deficient mice have shown that mice are protected from renal failure after ischemia/reperfusion (I/R) [31, 32], and that generation of the anaphylatoxin C5a [33] and the membrane attack complex (C5b-C9 or MAC) [32] may contribute to the pathogenesis of ischemic AKI. The proximal tubule is the primary damaged site after renal I/R; complement activation on the ischemic tubule is an important contributor to ischemic AKI. In addition, treatment with agents that inhibit the complement cascade at specific steps has proven effective at ameliorating ischemic AKI [33, 34]; and therapeutic targeting of classical and lectin pathways protects from ischemia-reperfusion-induced renal damage in animal model of kidney transplantation [35]. There is growing evidence that, in animal model of transplant kidney, complement plays a critical role in the acute induction of endothelial-to -mesenchymal transition, suggesting that therapeutic inhibition may be essential to prevent vascular damage and tissue fibrosis [36].

Complement activation in kidney occurs via the alternative pathway [31] and is independent of natural antibody [37].

Uncontrolled alternative pathway activation within the microvasculature is the primary cause of atypical haemolytic uremic syndrome (aHUS) [38]. The complement is also an important mediator of injury in ANCA-associated vasculitis [39] and antiglomerular basement membrane disease [40].

The MAC forms pores in cells resulting in cell activation. At high concentration, it causes cell death by lysis. Sublytic doses of MAC can activate renal parenchymal cells, which then release proinflammatory cytokines, reactive oxygen species, vasoactive chemicals, and profibrotic factors [41–44].

The aim of this study was to determine if MAC serum concentrations may allow clinicians to identify AKI-patients at high risk of adverse outcomes.

2. Material and Methods

2.1. Patients. We designed a case-control study that enrolled patients diagnosed with AKI in the Hospital del Mar in Barcelona, Spain, between October 2010 and December 2012. Patients were enrolled if they were at least 18 years of age and ADQI (Acute Dialysis Quality Initiative) serum creatinine criteria were met. Patients with chronic kidney disease were excluded. A control group included patients with the same clinical condition but normal renal function.

Case and control groups were classified according to the main etiology of AKI in four groups: septic group, ischemia-reperfusion group, nephrotoxicity group, and multifactorial group. The septic group included patients admitted in Intensive Care Unit (ICU) with sepsis or septic shock diagnosis with AKI (case group) or normal renal function (control group). Ischemia-reperfusion group included renal allograft recipients with delayed graft function (case group) or immediate good renal function (control group). Nephrotoxicity group included domiciliary hospitalization patients under colistin treatment with AKI (case group) or normal renal function (control group). Colistin is an antibiotic with a well-documented tubular toxicity [45]; domiciliary patients were enrolled, to avoid selection bias between septic and toxicity groups. Finally a multifactorial group was designed including patients with multiple AKI risk factors such as dehydration, contrast administration, and nonsteroidal anti-inflammatory therapy with AKI (case group) or normal renal function (control group).

AKI was defined according to the ADQI (Acute Dialysis Quality Initiative) criteria (RIFLE classification) [15, 16]. Briefly, patients were classified into the "risk" category if serum creatinine increased 1.5-fold, "injury" if serum creatinine increased 2-fold, and "failure" if serum creatinine increased 3-fold. The outcome criteria of loss of renal function and end-stage renal disease (ESRD) were defined by the duration of AKI.

Urine output criteria were not used in diagnosis or staging because these data were not available.

Patients were followed until time of hospital discharge or death and were staged according to the maximum increase in serum creatinine using the RIFLE classification.

For each individual patient, we recorded demographical data, past medical history, and laboratory data on admission and when AKI was solved.

This study adhered to the Principles of Helsinki Declaration, and the hospital's Ethics Committee (CEIC-IMAS) approved the study protocol, and all participants gave their written informed consent to participate in this study.

2.2. Plasma Samples and MAC Measurement. Samples were collected at the time of AKI diagnosis, except in ischemia-reperfusion group, in which samples were collected on the seventh day after transplantation, and when AKI was solved.

Whole blood samples were collected in EDTA tubes to prevent further *in vitro* complement activation. Samples were transported on ice and centrifuged to obtain plasma, and plasma was stored in aliquots at –80°C avoiding multiple freeze/thaw cycles.

Plasma samples were thawed at 37°C, and sandwich ELISAs were used to measure C5b-C9 membrane attack complex (MAC) according to the manufacturer's protocol (Hycult biotech). Briefly, samples and standards were incubated in microtiter wells coated with antibodies recognizing human MAC, streptavidin-peroxidase conjugate was used to bind the biotinylated tracer antibody, and the enzyme reaction was

stopped by the addition of oxalic acid. The absorbance at 450 nm was measured with a spectrophotometer, a standard curve was obtained by plotting the absorbance versus the corresponding concentrations of the MAC, and finally, the MAC concentrations were determined from the standard curve.

2.3. Statistical Analyses. Data are presented as mean (± standard deviation), absolute numbers, or percentages. Statistical significance was evaluated by using the Student's t-test or Paired t-test when it was required. Multiple-group comparisons were performed using ANOVA test, and univariate receiver-operating characteristic (ROC) curve analysis was performed to determine whether plasmatic MAC concentrations predicted AKI and the predictive value of MAC for AKI. ROC curves were considered statistically significant if the 95% CI of the area under the ROC curve (AUC) did not overlap 0.5. Statistical tests were performed in SPSS software, v 21, Chicago, IL, USA.

3. Results

3.1. Patient Characteristics. A total of 156 patients were included. Eighty one of them (52%) were diagnosed as having AKI and 75 (48%) were controls with normal renal function (non-AKI patients). Distribution according to the main AKI etiology was as follows: septic group ($n = 27$; AKI 13 patients (52%) versus non-AKI 14 patients (48%)), ischemia-reperfusion group ($n = 51$; AKI 29 patients (57%) versus non-AKI 22 patients (43%)), nephrotoxicity group ($n = 49$; AKI 21 patients (43%) versus non-AKI 28 patients (57%)), and multifactorial group ($n = 29$; AKI 18 patients (62%) versus non-AKI 11 patients (38%)). Distribution according to the main etiology is detailed in Figure 1.

All patients were Caucasian, mean age was 61.3 ± 15.2 years, and 102 (65%) were men. There were no statistically significant differences in demographic variables and past medical history data between AKI group and non-AKI group. Baseline characteristics of the study population are shown in Table 1.

For analysis, patients were assigned to their worst RIFLE category according to serum creatinine criteria. Fifteen patients (18.5%) were included in "risk" group; 13 patients (16%) were included in "injury" group; 33 patients (40.7%) were included in "failure" group; 20 patients (24.8%) were included in "loss" group, and nobody was classified in End-Stage-Renal-Disease (ESRD).

During hospitalization, 27 AKI patients needed renal replacement therapy; 21 of them recovered renal function, fully or partially, without the need to hemodialysis at time of discharge. In the AKI group (81 patients), at time of hospital discharge, 60 patients recovered partial or full renal function, 36 (23.1%) and 24 (15.4%), respectively.

3.2. Plasmatic Concentrations of C5B-C9 Complement Membrane Attack (MAC). Plasmatic MAC concentration was significantly higher in AKI-patients than in those with normal renal function (5848±83 versus 3702±52 mAU/mL, $P < 0.01$)

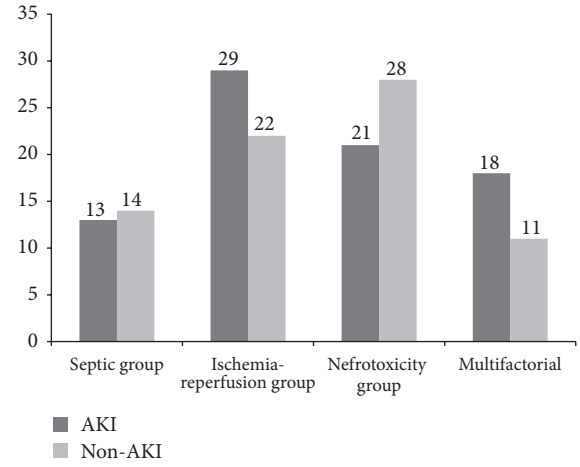

Figure 1: Distribution according to the AKI etiology, expressed in absolute numbers.

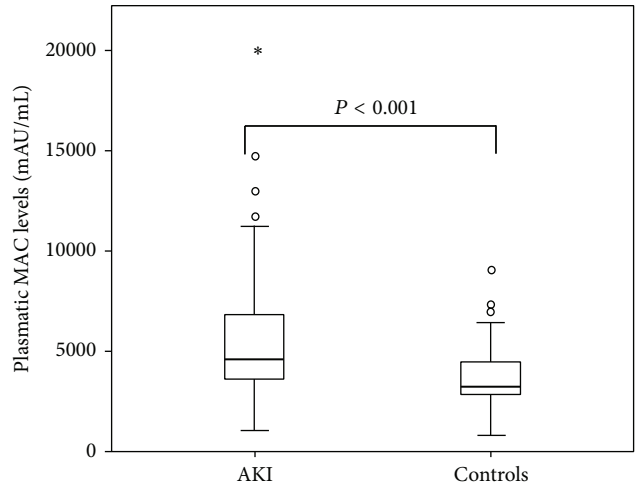

Figure 2: Comparison of MAC plasmatic concentrations between AKI patients and control patients.

(Figure 2). The difference is maintained independently of the AKI etiology (Table 2) and is proportional to the severity of AKI, measured by RIFLE classification; the mean value of MAC concentrations in "Risk" patients was 4905.4 mAU/mL and in "Injury" patients was 5246.3 mAU/mL, and the mean value of plasmatic MAC in "Failure" patients was 6971 mAU/mL, showing a progressive increase. In addition, correlation coefficient showed a direct relationship between serum creatinine and MAC plasmatic concentrations (Pearson coefficient 0.28, $P < 0.01$).

In AKI-patients, plasmatic MAC concentration showed a significant decrease when AKI was solved (5213 ± 685 versus 3402.5 ± 465 mAU/mL, $P < 0.02$). In contrast, plasmatic MAC of non-AKI patients did not show any change when the acute episode (sepsis, colistin administration, etc.) was solved.

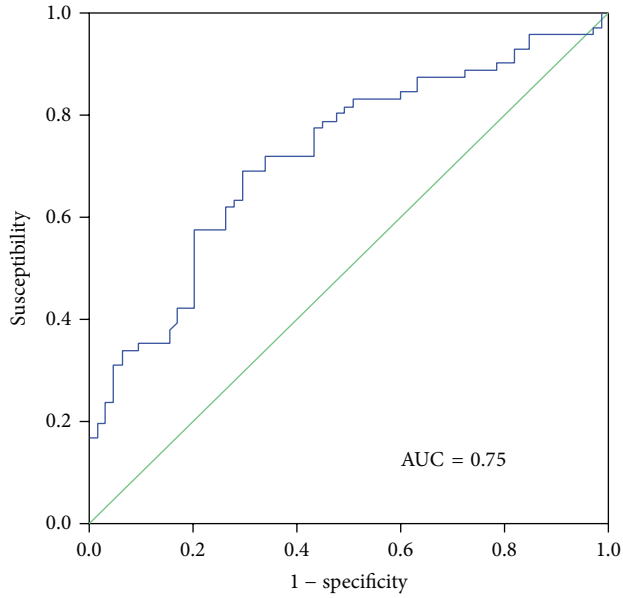

Figure 3: Univariate receiver-operating characteristic (ROC) curves for MAC plasmatic concentrations.

Table 1: Baseline characteristics of study population.

	AKI (n = 81)	Non-AKI (n = 75)
Age (years)	64.3 ± 14.3	61.5 ± 16.4
Male sex	50 (61.7%)	52 (69.3%)
Diabetes mellitus	21 (25.9%)	14 (18.7%)
Hypertension	55 (67.9%)	44 (58.7%)
Coronary artery disease	6 (7.5%)	16 (21.3%)
Mortality	17 (21%)	11 (14.7%)

AKI: Acute kidney injury.

Table 2: Plasmatic membrane attack complex concentrations and etiologies.

AKI Etiology	AKI	Non-AKI	P
Septic group	5407.5 ± 997.4	3743.4 ± 408.3	0.001
Ischemia-reperfusion group	8040 ± 816.4	4450.6 ± 382.7	0.001
Nephrotoxicity group	4906.6 ± 636.5	1278.7 ± 152.3	0.002
Multifactorial group	4720.6 ± 257	2312.3 ± 236.4	0.001

AKI: Acute kidney injury.

Plasmatic MAC concentration was significantly lower in AKI-patients who recovered renal function, compared with those patients who did not recover renal function at time of hospital discharge (5076.3 ± 277 versus 6146.8 ± 390 mAU/mL, $P < 0.001$).

In AKI-patients who died during hospitalization, we found significantly higher plasmatic MAC concentrations compared with AKI-patients who survived (7026±170 versus 5216 ± 240 mAU/mL, $P < 0.001$).

In contrast, differences in MAC concentrations between AKI-patients who needed renal replacement therapy compared with those who did not need hemodialysis during AKI episode did not reach statistical significance (6158.9 ± 527 versus 5699.2 ± 595 mAU/mL, $P < 0.07$).

The discrimination value of the plasmatic MAC concentration for AKI diagnosis was established by means of a receiver-operating characteristic (ROC) curve, with an area under the curve (AUC) of 0.75 (95% CI 0.64 to 0.86; $P < 0.001$) (Figure 3), and the cut-off value with the best sensitivity (75%) and specificity (70%) in predicting AKI was established in 3900 mAU/mL, positive predictive value 70%.

4. Discussion

Our data showed that complement pathway is activated in AKI, regardless the etiology of AKI, leading to the production of lytic complex C5b-C9 or MAC. Plasmatic MAC concentrations significantly decrease when AKI is solved. In addition, MAC concentrations were directly proportional to the severity of AKI, quantified by RIFLE classification.

These data agree with previous studies that showed complement activation as an important mechanism of renal injury in different diseases, aforementioned, aHUS [38], renal injury in ANCA-associated vasculitis [39], and antiglomerular basement membrane disease [40]. The proximal tubule is the primary site of injury after renal I/R, and complement activation on the ischemic tubule is an important cause of ischemic AKI [31]. C6-deficient mice, unable of generating MAC, are protected from renal I/R [32]. Given that complement activation is most prominent in the tubulointerstitium, the primary targets of MAC are likely tubular epithelial cells. MAC formation on the cell surface could contribute to cell necrosis, exacerbate adenosine triphosphate depletion (ATP) in hypoxic cells [46], and trigger intracellular signaling pathways and cell activation [47]. Fewer neutrophils infiltrated the kidneys in C6 deficient mice after I/R [32], which may be a direct effect of reduced inflammatory signaling. When complement is activated on cell or tissue surfaces, some of the formed MAC remains soluble (cytolytically inactive) and can increase adhesion molecule expression and promote neutrophil infiltration [48, 49] providing another potential link between complement activation on the tubular epithelial cells and renal inflammation.

In our study, plasmatic MAC concentrations identify AKI patients at risk of developing serious outcomes like death during hospitalization or unrecovered renal function at time of hospital discharge. Plasmatic MAC concentrations were high in AKI patients who needed renal replacement therapy, but these differences did not reach statistical significance, probably due to sample size.

The discrimination value of MAC concentrations was established by means of a ROC curve, and our results demonstrated that MAC AUC was not very different to that previously observed in NGAL studies [50].

Our study has several limitations. It is retrospective in nature, despite the fact that serum samples were prospectively collected. In our hospital there is not any important

Cardiac Surgery Department, and the I/R human model used was a renal allograft transplantation, which may add some immunological factors to the I/R derived injury. However, our results were consistent across the 4 types of AKI assessed.

In conclusion, plasmatic MAC concentration seems valuable as a marker of severity in AKI patients of different etiologies. Larger studies are needed to delineate its true power of discrimination.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Conversion to Sirolimus Ameliorates Cyclosporine-Induced Nephropathy in the Rat: Focus on Serum, Urine, Gene, and Protein Renal Expression Biomarkers

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Protocols of conversion from cyclosporin A (CsA) to sirolimus (SRL) have been widely used in immunotherapy after transplantation to prevent CsA-induced nephropathy, but the molecular mechanisms underlying these protocols remain nuclear. This study aimed to identify the molecular pathways and putative biomarkers of CsA-to-SRL conversion in a rat model. Four animal groups ($n = 6$) were tested during 9 weeks: control, CsA, SRL, and conversion (CsA for 3 weeks followed by SRL for 6 weeks). Classical and emergent serum, urinary, and kidney tissue (gene and protein expression) markers were assessed. Renal lesions were analyzed in hematoxylin and eosin, periodic acid-Schiff, and Masson's trichrome stains. SRL-treated rats presented proteinuria and NGAL (serum and urinary) as the best markers of renal impairment. Short CsA treatment presented slight or even absent kidney lesions and TGF-β, NF-$\kappa\beta$, mTOR, PCNA, TP53, KIM-1, and CTGF as relevant gene and protein changes. Prolonged CsA exposure aggravated renal damage, without clear changes on the traditional markers, but with changes in serums TGF-β and IL-7, TBARs clearance, and kidney TGF-β and mTOR. Conversion to SRL prevented CsA-induced renal damage evolution (absent/mild grade lesions), while NGAL (serum versus urine) seems to be a feasible biomarker of CsA replacement to SRL.

1. Introduction

Calcineurin inhibitors, such as cyclosporin A (CsA), are clinically important immunosuppressive drugs for prevention of allograft rejection after organ transplantation and also for several autoimmune disorders, such as psoriasis, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease, among other indications [1, 2]. Despite the impressive reduction in the number of acute rejection episodes after the beginning of CsA use in clinical practice, long-term therapy is typically associated with drug-induced nephrotoxicity [3]. Renal dysfunction is an independent risk factor for graft loss and mortality after kidney transplantation (KTx) and cardiovascular disease (CVD) is the main cause of dead post-KTx [4–6]; thus, extended long-term graft survival has not been completely achieved.

Complete avoidance of CNIs, in particular of CsA, from transplantation immunotherapy, has been viewed as an invalid option by almost all the transplantation centres worldwide, particularly because of the risks in acute rejection. The main long-term goals of immunosuppressive therapy are to reduce drug exposure while maintaining a well-functioning graft, keeping efficacy and minimizing drug-induced serious side-effects, including infections and nephrotoxicity [7]. The recognition of these serious adverse effects sparked interest in CsA-sparing strategies [8]: dose reduction is associated with a modest improvement in renal function, but CsA-induced nephropathy is progressive over time when exposure is maintained; CsA avoidance is associated with high acute rejection rates and is not an option; minimization protocols are the current preferred therapy, including the conversion from CsA to other drugs, especially sirolimus (SRL), an inhibitor of the mammalian target of rapamycin (mTOR) [9–11]. Despite the SRL-evoked proteinuria, this drug has been indicated as a less nephrotoxic immunosuppressive agent per se when compared with calcineurin inhibitors [12], but its use after CsA would have an impact on the renal function/structure that should be precisely defined. The major question nowadays concerning the protocols of immunotherapy is to find the most adequate duration for CsA exposure and the proper moment for replacement by other less nephrotoxic drugs, such as SRL, in order to afford renoprotection without compromising the graft by a rejection episode.

Early diagnosis of nephropathy can greatly improve patient diagnosis, but the initial stages of CsA-induced nephropathy are largely asymptomatic, making early diagnosis difficult [13]. Since the current diagnostic techniques employed to detect CsA nephropathy seem to be unsatisfactory, the identification of novel, early disease indicators is currently a major research focus. Identifying drug safety liabilities or predictive biomarkers for drug-induced organ damage is of great value. Drug safety evaluation has mainly been based on biochemical and histopathological data, but transcriptional profiling has the promise of being able to detect toxicity objectively. In addition, gene expression changes associated with toxicity may also accurately and earlier assist our understanding on the mechanism of certain drug-induced toxicity [14, 15], which will be pivotal for drugs with a low therapeutic window, such as the immunosuppressive agents. The precise mechanisms and biomarkers, underlying transition from renal dysfunction to nephrotoxicity, deserve better elucidation; experimental studies have been important to improve the knowledge on this translational issue of clinical relevance.

The mechanisms underlying CsA-induced nephropathy have been debated for the last decades and are clearly viewed as having a multifactorial nature (including vasorelaxant/vasoconstrictor disequilibrium, oxidative stress, apoptosis, and proliferation/fibrosis) [16–20], as evolving and changing with the increased duration of exposure [3, 21, 22] and as modulated by influence on renal tissue gene expression [23–25]. With nephrotoxicity remaining a major contributing factor to late allograft damage, it is crucial to understand the impact on the kidney tissue of protocols of conversion from CsA to SRL and identify early biomarkers in order to improve the therapeutic strategies after transplantation, thus extending long-term graft survival by reducing cardiorenal mortality. Molecular studies on animal tissues are essential to elucidate these questions and emergent biomarkers of renal damage, such as NGAL, KIM-1, and CTGF, among others, would increase our knowledge of how to better manage this drug-related nephropathy.

We have previously shown, in a preliminary study using an experimental model for comparing CsA and SRL, that distinct mechanisms and players are involved in the effects of these drugs on the kidney tissue, in a moment when renal lesions are almost absent [26]. In addition, our previous data suggested that early conversion from CsA to SRL promotes a better cardiorenal profile than late conversion [27], whose mechanisms and biomarkers deserve now more elucidation. The current study intended to clarify the pathways of nephropathy evolution in a protocol of conversion from CsA to SRL in the rat, focusing on serum, urine, and renal (gene and protein) tissue samples, as well as to elucidate the involvement of several emergent biomarkers of renal damage which are putative candidates to act as players in the evolution from renal dysfunction to nephrotoxicity.

2. Materials and Methods

2.1. Animals and Treatments. Male Wistar rats (aged 11 weeks) were purchased from Charles River Laboratories (Barcelona, Spain) and housed (two animals per cage) in IVC racks, subjected to 12 h dark/light cycles and given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Forty-two animals were divided into two groups, according to the period of the treatments. Eighteen animals were used in a protocol of 3-week treatments and divided in 3 groups: control (vehicle), cyclosporin A (5 mg/Kg/day of Sandimmun Neoral, Novartis Farma Produtos Farmacêuticos SA, Sintra, Portugal), and sirolimus (1 mg/kg BW/day of Rapamune, Laboratórios Pfizer Lda., Lisbon, Portugal). Twenty-four animals were used in a protocol of 9-week treatments and divided into 4 groups: control (vehicle), cyclosporin A, sirolimus, and conversion (cyclosporin A during 3 weeks and sirolimus during the last 6 weeks). Treatments were performed by oral gavage and body weight was monitored daily. Animal experiments were conducted according to the European Council Directives on Animal Care and to the National Authorities.

2.2. Sample Collection and Preparation. The rats were anesthetized (i.p.) with 2 mg/Kg BW of a 2 : 1 (v : v) 50 mg/mL Ketamine (Ketalar, Parke-Davis, Pfizer Laboratories Ltd, Seixal, Portugal) solution in 2.5% chlorpromazine (Largatil, Rhône-Poulenc Rorer, Vitória laboratories, Amadora, Portugal). When the animal did not present response to stimulus, blood samples were immediately collected by venipuncture from the jugular vein in needles with no anticoagulant for serum samples collection. Then, the rats were sacrificed by cervical dislocation, and the kidneys were immediately removed, weighted, divided, and stored according to the next procedure: RNA-stabilizer reagent for gene expression

determinations, frozen in nitrogen for lipid peroxidation assays, prefixed with formaldehyde for histopathological analysis and immunohistochemical detections.

2.3. Serum and Urinary Measures

2.3.1. Serum Biochemical Data. Serum creatinine and blood urea nitrogen (BUN) contents were evaluated by automatic validated methods and equipment (Hitachi 717 analyser, Roche Diagnostics Inc., MA, USA). Serum levels of interleukin 1β (IL-1β), interleukin 2 (IL-2), vascular epidermal growth factor (VEGF), and transforming growth factor beta 1 (TGF-β_1) were measured by ultrasensitive Quantikine ELISA kits (R&D Systems, Minneapolis, USA). High sensitivity CRP (hsCRP) was detected by using an ELISA kit (Alpha Diagnostic International, San Antonio, USA). Interleukin-7 (IL-7) was measured through an ELISA kit obtained from Wuhan EIAAB Science Co (Wuhan, China).

2.3.2. Urinary Data. The animals were housed in metabolic cages during 24 hours and received tap water and food *ad libitum*. The urine concentration of creatinine, BUN, and protein was assessed in 24-hour urine (Cobas Integra 400 plus, Roche), and the urine volumes were measured in order to calculate creatinine and BUN clearance and glomerular filtration rate, as previously described [28].

2.3.3. Serum, Kidney, and 24-Hour Urine Lipid Peroxidation. Lipid peroxidation was determined by assaying the malondialdehyde (MDA) production by means of the thiobarbituric acid (TBA) test. Briefly, 100 μL of kidney tissue supernatant, serum, or urine (previously centrifuged to remove particulates) was incubated 1 hour in a TBA solution. Samples incubated at 90°C for 60 min. In this test, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment producing maximal absorbance at 532 nm. The concentration of MDA was calculated with respect to a calibration curve using 1,1,3,3-tetramethoxypropane as the external standard (range: 0.1–83.5 μM) and results were expressed as μM/g of kidney tissue and μM of serum or urine.

2.4. RT-qPCR Kidney Gene Expression

2.4.1. Total RNA Isolation. The kidneys were stored in RNA later solution (Ambion, Austin, TX, USA). For RNA extraction, 10 mg of tissue was weighted, 450 μL of RLT lysis buffer was added, and tissue disruption and homogenization for 2 min at 30 Hz were performed using a TissueLyser (Qiagen, Hilden, Germany). Tissue lysates were processed according to the RNeasy Mini Kit protocol (Qiagen, Hilden, Germany). Total RNA was eluted in 50 μL of RNase-free water (without optional treatment with DNAse). In order to quantify the amount of total RNA extracted and to verify RNA integrity (RIN, RNA Integrity Number), samples were analyzed using a 6000 Nano Chip kit, in the Agilent 2100 Bioanalyzer (Agilent Technologies, Walbronn, Germany) and the 2100 expert software, following manufacturer's instructions. The isolation yield was from 0.5 to 3 μg; RIN values were 6.0–9.0 and purity (A260/A280) was 1.8–2.0.

2.4.2. Reverse Transcription. RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA). One microgram of total RNA was mixed with a 2x First-Strand Reaction Mix and a SuperScript III Enzyme Mix (Oligo (dT) plus random hexamers). Reactions were carried out in a thermocycler Gene Amp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA), 10 min at 25°C, 30 min at 50°C, and 5 min at 85°C. Reaction products were then digested with 1 μL (2 U) RNase H for 20 min at 37°C and, finally, cDNA was eluted to a final volume of 50 μL and stored at −20°C.

2.4.3. Relative Gene Expression Quantification. Gene expression was performed using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, USA). A normalization step preceded the gene expression quantification, using geNorm Housekeeping Gene Selection kit for *Rattus norvegicus* (Primer Design, Southampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Ghent, Belgium) to select optimal housekeeping genes for this study [29]. Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen, Hilden, Germany) with optimized primers for transforming growth factor beta 1 (QT00187796), proliferating cell nuclear antigen (QT00178647), mechanistic target of rapamycin (QT00180586), nuclear factor kappa B (QT01577975), monoclonal antibody Ki-67 (QT00450786), and tumor protein p53 (QT00193522) as proliferative markers; vascular endothelial growth factor beta (QT01290163) as angiogenic marker; interleukin 1 beta (QT00181657), interleukin 2 (QT00185360), tumor necrosis factor (QT00178717), cyclooxygenase 2 (QT00192934), and C-reactive protein (QT00391650) as inflammatory markers. Endogenous controls were used for kidney [glyceraldehyde-3-phosphate dehydrogenase (QT00199633), actin beta (QT00193473), and topoisomerase I (QT01820861)]. A QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used according to manufacturer's instructions. RT-qPCR reactions were carried out with 100 ng cDNA sample, primers (50–200 nM), and 1x QuantiTect SYBR Green PCR Master Mix. Nontemplate control reactions were performed for each gene, in order to assure nonunspecific amplification. Reactions were performed with the following thermal profile: 10 min at 95°C plus 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Real-time PCR results were analyzed with SDS 2.1 software (Applied Biosystems, Foster City, USA) and quantification used the $2^{-\Delta\Delta Ct}$ method [30]. The results were obtained in CNRQ (calibrated normalized relative quantities).

2.5. Histopathological Analysis

2.5.1. Haematoxylin and Eosin Staining. Samples were fixed in Bock's fixative and embedded in paraffin wax, and 4 μm thick sections were mounted on glass slides and stained for

routine histopathological diagnosis with haematoxylin and eosin (H&E).

2.5.2. Periodic Acid of Schiff Staining. Periodic acid of Schiff (PAS) was used to evaluate and quantify the renal lesions. Samples were fixed in 10% neutral formalin, embedded in paraffin wax, and 4 μm thick sections were immersed in water and subsequently treated with a 1% aqueous solution of periodic acid, then washed to remove any traces of the periodic acid, and finally treated with Schiff's reagent. All samples were examined by light microscopy using a Zeiss Microscope Mod. Axioplan 2. The degree of injury visible by light microscopy was scored in a double-blinded fashion by two independent pathologists. Lesions were evaluated on the total tissue on the slide.

2.5.3. Analysis of Lesions. Glomerular damage was assessed by evaluating mesangial expansion, the glomerular basement membrane and the Bowman's capsule thickenings, nodular sclerosis, and vascular pole hyalinosis. The analysed tubulointerstitial lesions comprised inflammatory infiltration, presence of hyaline cylinders, tubular basement membrane irregularity, tubular calcification, tubular vacuolization, and the association of interstitial fibrosis and tubular atrophy (IFTA). The evaluation of vascular lesions was concentrated on vascular congestion and hyperemia, arteriolar vacuolization, arteriolosclerosis, and arteriosclerosis. A semiquantitative rating for each slide ranging from normal (or minimal) to severe (extensive damage) was assigned to each component. Severity was graded as absent/normal (0), mild (1), moderate (2), and severe (3). Scoring was defined according to the extension of the lesion (number of capsules): normal: 0%; mild: <25%; moderate: 25–50%; severe: >50%. The final score of each sample was obtained by the average score observed in the individual glomeruli, in the considered microscopic fields. Tubular calcification was evaluated and graded by the same semiquantitative method. Regarding vascular lesions, arteriosclerosis was scored as 0 if no intimal thickening was present, as 1 if intimal thickening was less than the thickness of the media, and as 2 if intimal thickening was more than the thickness of the media and considering the worst artery on the slide. Using PAS, the rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining), respecting tissue specificity scoring when adequate.

2.5.4. Masson's Trichrome Staining. Deparaffinise and rehydrate through 100% alcohol, 95% alcohol, and 70% alcohol. Wash in distilled water. After that, refix in Bouin's solution for 1 hour at 56°C to improve staining quality and rinse in running tap water for 5–10 minutes to remove the yellow colour. Stain in Weigert's iron hematoxylin working solution for 10 minutes and rinse in running warm tap water for 10 minutes. Wash in distilled water. Stain in Biebrich scarlet-acid fuchsin solution for 10–15 minutes and wash in distilled water. Differentiate in phosphomolybdic-phosphotungstic acid solution for 10–15 minutes and transfer sections directly to aniline blue solution and stain for 5–10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2–5 minutes and wash in distilled water. Finally, dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol, clear in xylene, and mount with resinous mounting medium. All samples were examined in a blind fashion by expert personnel (pathologists) by light microscopy using a Zeiss Microscope Mod. Axioplan 2.

2.6. Immunohistochemical Analysis. Immunohistochemical analyses were performed in 4 μm thick sections with sagittal orientation of kidney fixed in Bock's fixative and embedded in paraffin wax. The samples were processed by indirect immune detection technique with mouse and rabbit specific HRP/DAB detection IHC kit (Abcam, Cambridge, UK) using the primary antibody mammalian target of rapamycin (Millipore Corporation, Billerica, MA, USA, 04-385) (dilution 1 : 250). The protocol was executed according to the manufacturer's instructions. In this study, we employed primary antibodies against CTGF (dilution 1 : 100, ab6992; Abcam), TGF-b (dilution 1 : 100, ab66043; Abcam), mTOR (dilution 1 : 250, 04-385; Millipore), NF-$\kappa\beta$ p50 (dilution 1 : 500, sc-114; Santa Cruz Biotechnology), and KIM-1 (dilution 1 : 14, AF3689; R&D Systems). For KIM-1 detection the secondary antibody was anti-goat (dilution 1 : 500, sc2771; Santa Cruz Biotechnology). After testing the different antigen-retrieval methods and negative controls, immunohistochemical procedures were optimized. To identify PCNA protein we used a standard kit (ready to use, 93-1143, Invitrogen Corporation). An appropriate positive control was used in each staining run, and each slide was stained with a negative control obtained by omitting the primary antibody. Standard procedures were used for visualisation and the staining was quantified using a semiquantitative scale (1–4) that evaluated both the intensity and area of staining. Intensity was graded as very low (1), low (2), moderate/mild (3), and high (4); staining area was graded as <25% (1), 25–50% (2), 25–75% (3), and >75% (4). All slides were reviewed independently by 2 investigators blinded to the data. In this evaluation a quantitative immunohistochemical score (QIC) was calculated. QIC = % of staining area * staining intensity * 0.1.

2.7. Statistical Analysis. Statistical analyses were performed using the GraphPad Prism for Windows (version 5.00). The results are presented as means ± S.E.M. Comparisons between groups were performed using one-way ANOVA test, followed by the post hoc Bonferroni's multiple comparisons. The association between categorical variables was analyzed using Pearson's test in the IBM Statistical Package for Social Sciences (SPSS) for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at P less than 0.05.

3. Results

3.1. Kidney Histomorphological Changes and Collagen Deposition. Nephrotoxicity was confirmed by two independent pathologists, which have characterized the lesions through

the attribution of degrees to each vascular, glomerular, and tubular lesion, examining kidney slices stained with H&E and PAS. After 3 weeks of CsA treatment, only slight morphological changes on the tubules (tubular vacuolization) were found when compared with the control (Figure 1). However, signs of toxicity were identified in the vessels since some kidney slices revealed arteriolar vacuolization and hyperemia. SRL treatment during identical period, described in the literature as less nephrotoxic than CsA, surprisingly revealed some lesions in the vascular (congestion and hyperemia) and tubular (vacuolization and calcification) fields. Total lesion scoring showed that only SRL was able to induce significant damage in the vessels ($P < 0.05$) and tubules ($P < 0.01$) after the first 3-week period (Figures 2(a_1) and 2(c_1)). Long-term CsA treatment (9 weeks) promoted relevant changes on the kidney (vessels, glomeruli, and tubules) structure, which are viewed as clear signs of nephrotoxicity. The main changes encountered compared with the normal controls are represented in Figure 1. In the long-term CsA exposure, vascular congestion, vascular hyperemia, and arteriolar vacuolization and arteriolosclerosis were identified, being all statistically significant versus the control group (Figure 1(a_{1-4})). Sirolimus revealed similar pattern to that found for CsA but does not induce arteriolosclerosis, compared to CsA ($P < 0.05$). The conversion protocol does not promote any advantage in the vascular field when compared with the CsA treatment alone; two rats of the group presented arteriosclerosis (grades 1 and 2).

Regarding the glomerular field after 9 weeks of CsA treatment, the major lesions found were mesangial expansion ($P < 0.01$), hyalinosis of vascular pole ($P < 0.001$), and thickening of Bowman's capsule ($P < 0.001$) when compared with the control rat kidneys (Figure 1(b_{1-4})). One rat presented hydronephrosis and cortical atrophy. In the SRL treatment the single significant lesion found was Bowman's capsule thickening, confirming a better profile in the conversion protocol. Mesangial expansion and vascular pole hyalinization grade were almost absent when compared with the isolate CsA treatment. However, all rats from the conversion group showed glomerular basement membrane thickening ($P < 0.05$ versus CsA). The global glomerular score clearly showed that SRL is less toxic than CsA ($P < 0.01$) and this was reflected in the lower score found in the conversion group.

CsA induces tubular damages and the main lesions identified were tubular vacuolization and calcification ($P < 0.001$, both), versus the normal profile found in the control rats (Figures 1(c_{1-4}) and 2(c_{2-3})). However, the presence of hyaline cylinders and inflammatory infiltration was identified in almost all the kidneys (grade 1, less than 25% of the tubules). Sirolimus treatment only induced tubular vacuolization. However, when CsA was used prior to SRL (conversion group), tubular calcification and vacuolization remain present in the same grade than that encountered for CsA therapy alone. In contrast, hyaline cylinders ($P < 0.05$) and inflammatory infiltration were absent in the kidneys of the conversion group rats. Figure 2(c_1) gives an idea about the treatments influence on the tubules. Clearly, CsA promotes more tubular damage than SRL, and CsA conversion to SRL revealed less total lesions grade in glomerular and tubular fields.

Collagen is the major insoluble fibrous protein in the extracellular matrix and in connective tissue and is clearly marked with Masson's trichrome; modifications of collagen production reflect cellular changes and consequent kidney dysfunction. In the kidneys from vehicle-treated rats, collagen staining was rare in the glomeruli, and a small amount of blue Trichrome staining appeared in the outer borders tubules and around the vessels (Figures 2(a_{4-5}), 2(b_{4-5}), and 2(c_{4-5})). After 9 weeks of CsA treatment, staining was clearly visible in the outer borders of tubular cells (cortex and medulla), well representing wide-spread interstitial fibrosis. Bowman's capsule thickening also occurred in some glomeruli; around the vessels we also verified higher collagen deposition in the CsA-treated rats. Sirolimus and conversion group revealed normal collagen staining (comparable to that encountered in the control group).

3.2. Nephrotoxicity Evaluation through Serum, Kidney, and Urine Markers. Classical serum markers of renal function, such as creatinine and BUN, presented a trend to increased levels after 3 weeks of CsA treatment, accompanied by a trend to decreased creatinine and BUN clearances (Figures 3(a), 3(b), 3(d), and 3(e)), resp.); however, all those measures did not reach a statistical significant value. Moreover, CsA showed a trend to decreased glomerular filtration rate (GFR). On the other hand, unchanged values were found for the SRL group for all serum and urine markers. Long-term CsA treatment (9 weeks) presented a trend to aggravated serum creatinine and BUN levels; in addition, while GFR and kw/bw remained decreased, kidney TBARs production and clearance significantly increased ($P < 0.05$). The main change found for the SRL treatment after 3 weeks was increased urinary protein, with additional increment after 9 weeks, suggesting a time-dependent effect. The conversion protocol revealed no significant change on serum creatinine and BUN levels and clearances; moreover, GFR remains unchanged as well as urinary protein and TBARs (Figure 3).

NGAL has been described as a putative biomarker of nephrotoxicity. Long-term CsA treatment only promoted serum NGAL increment ($P < 0.05$), without affecting urine and clearance values. The rats treated with SRL presented increased serum and urine NGAL contents, as well as augmented clearance. The use of SRL to replace CsA leads to increased NGAL levels in serum and urine compared to the CsA group ($P < 0.05$) (Figures 4(a), 4(b), and 4(c)). Figure 4(d) showed the correlation between serum and urine NGAL levels; interestingly, with only 6 samples of each group, while the control and CsA groups were unable to show significant Person's correlation; in SRL group there was a good linearity ($r = 0,627$, $P = 0,183$), which was even more evident and statistically significant, in the conversion group ($r = 0.905$, $P = 0.034$).

3.3. Serum Markers of Inflammation, Proliferation, and Angiogenesis. Unchanged values of serum hsCRP were found for both immunosuppressive drugs when compared with

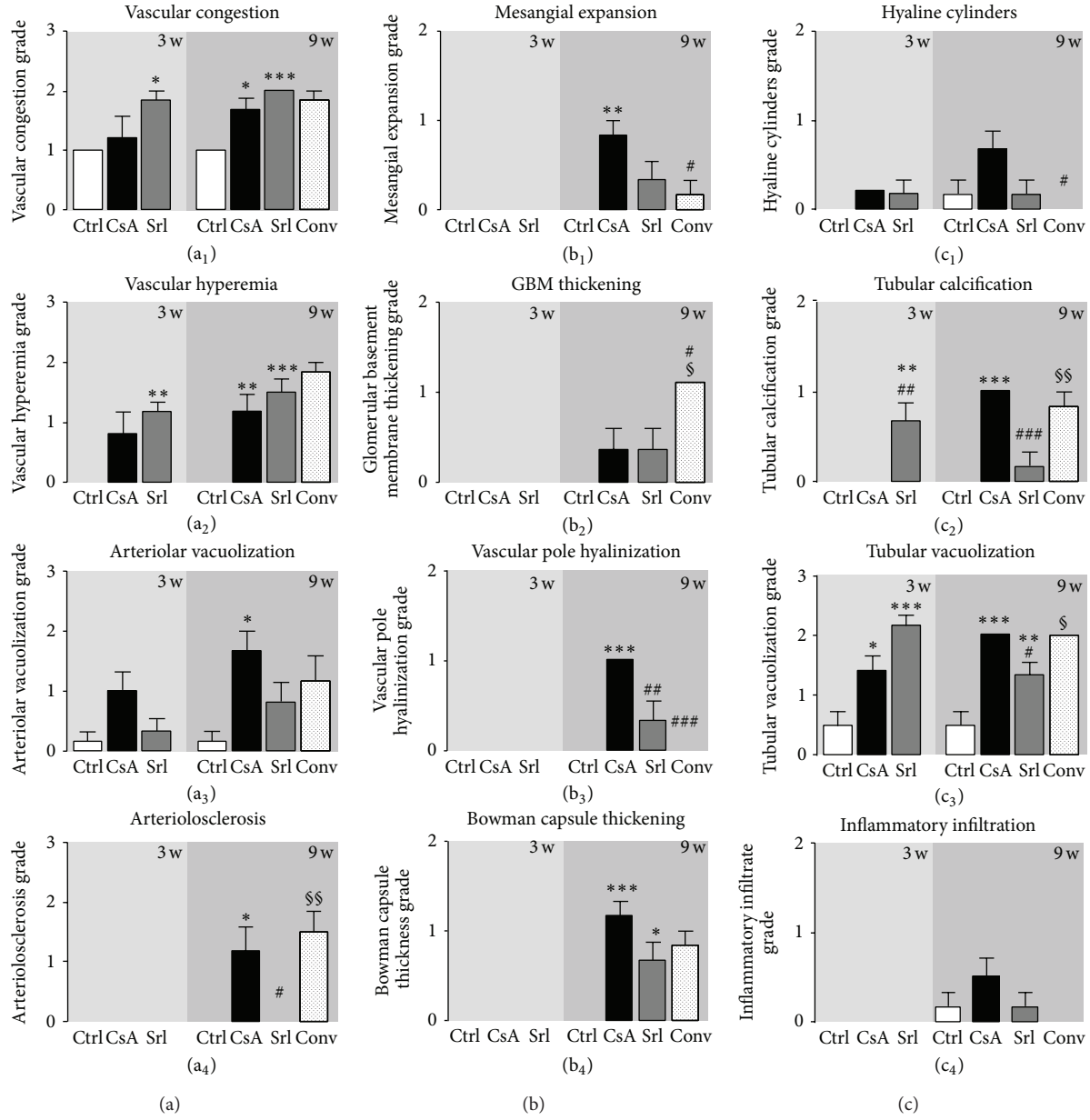

Figure 1: Semiquantitative evaluation of vascular (a), glomerular (b), and tubulointerstitial (c) lesions. Each graphic represents one lesion for the 3 groups at week 3 (control, cyclosporin A, and sirolimus) and 4 groups at week 9 (control, cyclosporin A, sirolimus, and conversion). Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus cyclosporin; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ versus sirolimus. GBM thickening, glomerular basement membrane thickening.

the control group. However, serum IL-1β showed distinct patterns; in fact, SRL treatment was able to increase IL-1β levels in the short-term treatment and CsA to decrease in the long-term use (Figures 5(a) and 5(b)). Serum IL-2 levels, which is simultaneously a marker of inflammation and immunosuppressive activity, decreased after 3 weeks of SRL treatment ($P < 0.01$). Identical reduction ($P < 0.01$) was found at 9 weeks for all the treated groups (CsA, SRL, and conversion) versus the control one (Figure 5(c)). Serum contents of the VEGF only decreased in the short-term treatment for both drugs, with unchanged values in long-term protocols. Serum TGF-beta levels showed a trend to increased values after 3 weeks, which was even more pronounced after 9 weeks in the CsA group (Figures 5(e) and 5(f)). Interestingly, similar pattern was encountered for serum IL-7 levels, showing significant correlation with serum TGF-β_1 contents in the short- and long-term treatments ($r = 0.871$, $P = 0.129$; $r = 0.873$, $P = 0.053$, resp., in Person's Test).

3.4. Kidney Gene Expression Evaluation. Several markers of proliferation, fibrosis, inflammation, and angiogenesis were evaluated in terms of kidney mRNA expression in the weeks 3 and 9 for the three immunosuppressive protocols in comparison to control group (Figure 6). After 3 weeks of CsA treatment, a significant downregulation of the antigen identified by the monoclonal antibody Ki67 (MKi67) ($P <$

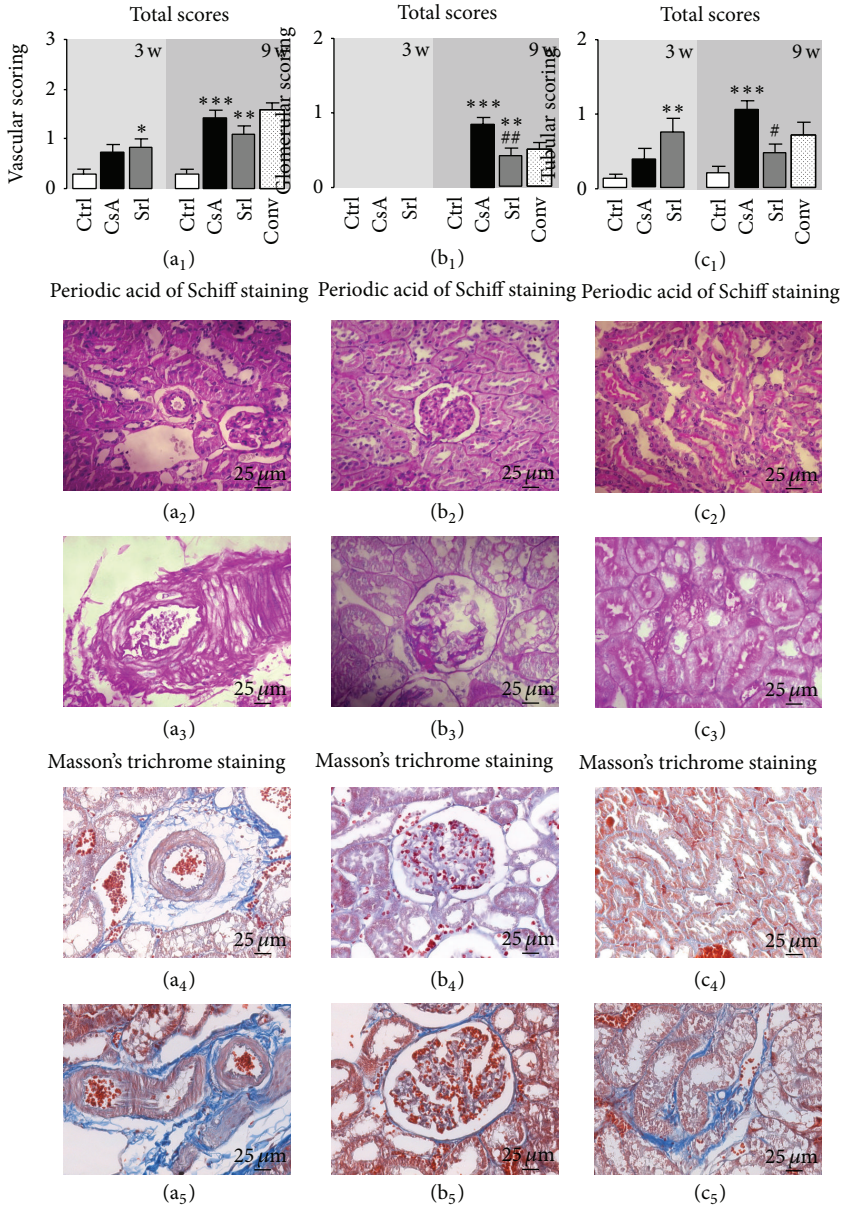

FIGURE 2: Total score of vascular (a_1), glomerular (b_1), and tubulointerstitial (c_1) lesions and representative photomicrographs of kidney histomorphologic sections with PAS and Masson's trichrome stainings. The image (a_2) represents a normal kidney arteriole from the control group and A_3 an arteriolosclerosis lesion present in all the rats treated with CsA. (b_2) and (b_3) represent a normal capsule and a vascular pole hyalinization and Bowman's capsule thickening from the CsA group, respectively. (c_2) and (c_3) images match normal tubules and tubular calcification in the kidney of CsA-treated rats, respectively. Representative photomicrographs of kidney histomorphologic sections with Masson's trichrome staining for control ((a_4), (b_4), and (c_4)) and CsA ((a_5), (b_5), and (c_5)). CsA promotes collagen fibers deposition around arterioles, Bowman's capsules, and tubules (fibrosis). Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus cyclosporin.

0.001), CRP ($P < 0.01$), TNF-alpha ($P < 0.05$), and VEGF ($P < 0.01$) was found (Figure 6). However, IL-2, COX-2, mTOR, and IL-1β remain unchanged. Furthermore, there was a significant overexpression of proliferating cell nuclear antigen (PCNA) and tumor protein p53 (TP53) mRNA ($P < 0.001$), accompanied by a slight increase ($P < 0.05$) in the expression of TGF-β_1 and NF-κB. On the other hand, the mTOR inhibitor only stimulated the expression of TP53 gene and downregulated some inflammatory markers (TNF-α, COX-2, and IL-1β). In the long-term CsA treatment, almost all the genes presented normal mRNA expression, when compared with the control group. However, a significant upregulation of IL-2, mTOR, and Mki67 was encountered. In the SRL and conversion groups only mTOR and Mki67 remained overexpressed, in contrast to what was observed with the short-term exposures.

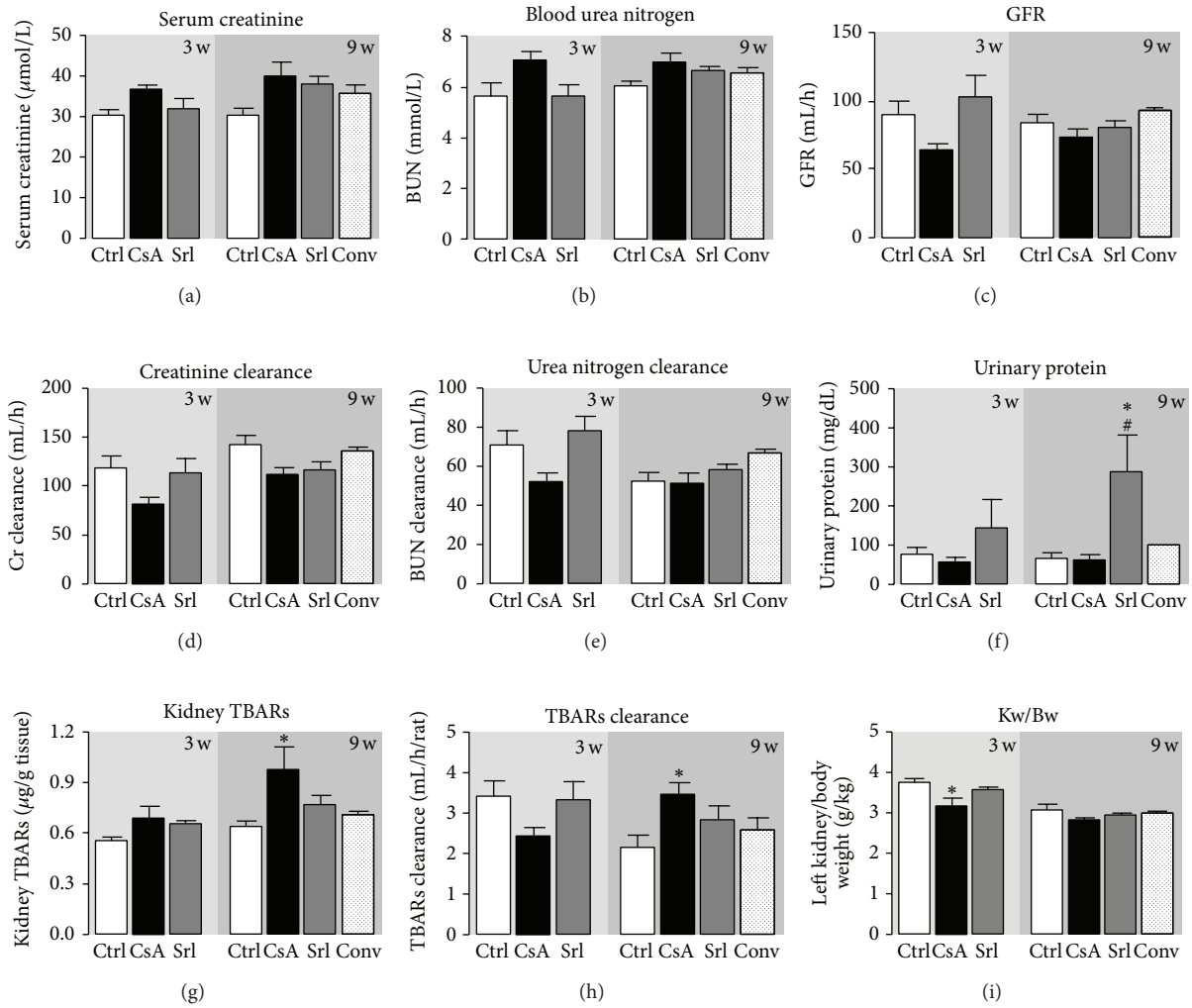

FIGURE 3: Serum, urine, and kidney markers of renal function. Creatinine serum levels (a) and clearance (d), blood urea nitrogen levels (c) and clearance (f), glomerular filtration rate (c) and urinary protein (f), kidney lipid peroxidation (malondialdehyde levels) (g), malondialdehyde clearance (h), and kidney weight/body weight ratio (i), at week 3 and week 9 for all treatments. Values are mean ± SEM. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus cyclosporin.

3.5. Kidney Protein Expression Evaluation. Short-term CsA treatment increased CTGF, KIM-1, mTOR, NF-$\kappa\beta_1$, and TGF-β ($P < 0.001$) protein expression, when compared to control, while SRL treatment was unable to promote changes on the expression of these proteins (Figures 7 and 8). Long-term treatment with CsA promoted increased expression of mTOR, TGF-β, and CTGF, versus the control group, but the last two proteins present less area and stain intensity (QIC score) when compared to the short-term (3 weeks) CsA treatment. Kidney KIM-1, NF-$\kappa\beta_1$, and PCNA expression were unchanged in the CsA-treated rats when compared with the control animals. SRL treatment promoted, after 9 weeks, a decreased KIM-1 expression and overexpression of TGF-β. In the conversion protocol, CTGF and PCNA protein overexpression was obtained, but mTOR and TGF-β expression were significantly reduced when compared with the CsA group (Figures 7 and 8).

4. Discussion

Monitoring immunosuppressive therapy in solid organ transplant patients is based on measuring putative indicators of allograft rejection, as well as on regularly assessing drug blood levels, which should be maintained within the established therapeutic range for the drug in order to maintain immunosuppressive efficacy without excessive/undesirable side-effects. Drug-related nephrotoxicity evaluation has been mainly based on classical serum measures of renal function, which are easier to perform and less expensive; however, an increasing amount of evidence suggests that these markers cannot accurately reflect the renal function status at a given time point of drug use. In fact, traditional markers of nephrotoxicity, such as increased BUN or serum creatinine, have been reported as insensitive, only indicating damage when 70–80% of renal epithelial mass has been lost [31, 32]. The use of noninvasive samples (e.g., urine) has been pointed

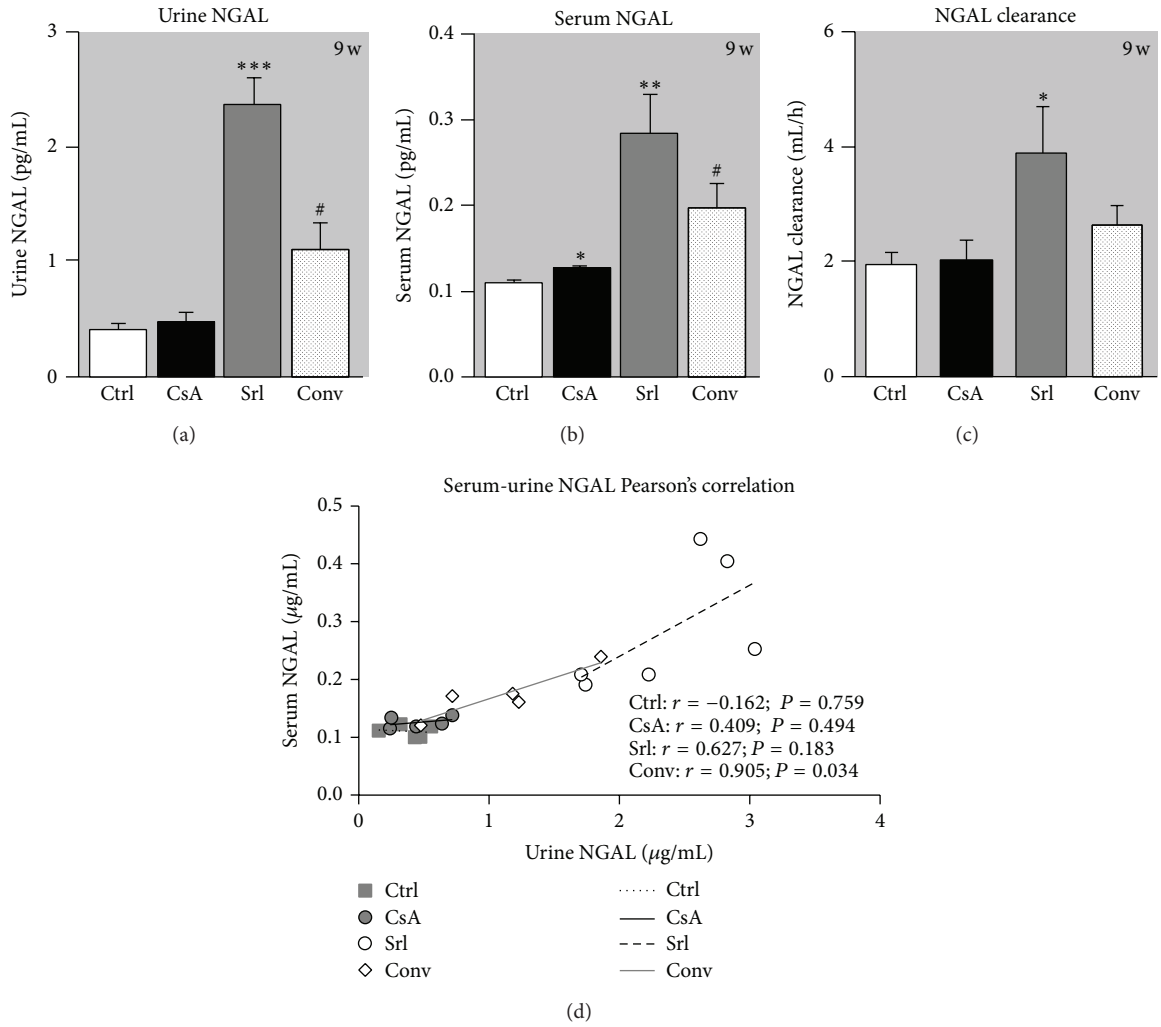

Figure 4: Serum and urine NGAL correlation as kidney marker of toxicity. Urine (a), serum NGAL (b), and clearance (c) at week 9. Values are mean ± SEM. Correlation and P value of urine and serum NGAL are shown in (d). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$ versus cyclosporin A.

as a choice to access drug-related toxicity; however, detection of enzymes and other proteins can be difficult due to their instability and high variability levels in urine [32]. Identifying potentially useful biomarkers in peripheral blood and urine, compared to kidney tissue markers (gene or protein), will be clinically very important. The current study was intended to clarify the pathways of nephropathy evolution in a protocol of conversion from CsA to SRL in the rat, focusing on serum, urine, and renal (gene and protein) tissue samples, as well as to elucidate the involvement of several emergent biomarkers of renal damage which are putative candidates to act as players in the evolution from renal dysfunction to nephrotoxicity.

In our study, the classical serum and urine markers were unable to accurately reflect the changes on renal function after both the short- and long-term treatments, despite the presence of renal lesion, which were more pronounced for the longer CsA exposure; that failure demonstrates the need of better biomarkers of renal dysfunction/damage. Regarding renal pathology characterization, we found that vessels are the first renal structures affected by CsA use, after just 3 weeks of treatment, as shown by the presence of some lesions, such as vascular hyperemia and arteriolar vacuolization (that might be related to hypertension appearance); the lesions were further aggravated with prolonged CsA exposure. This data complements the information that the first CsA pathologic events are related to afferent arteriolar vasoconstriction, thrombotic microangiopathy, and isomeric tubular vacuolization [33]. In addition, acute CsA events are related to decreased vasodilation and unopposed vasoconstriction and free radical formation, which are among the main mechanisms underlying development of hypertension and decreased GFR [34]. SRL has been described as a less nephrotoxic agent than CsA [20], which explains the fact that mTOR inhibitors have been used to replace CsA [9, 11]. According to our data, SRL induces less toxicity in vascular, glomerular, and tubular fields than CsA, and this factor leads to a better profile in the conversion group; however, tubular

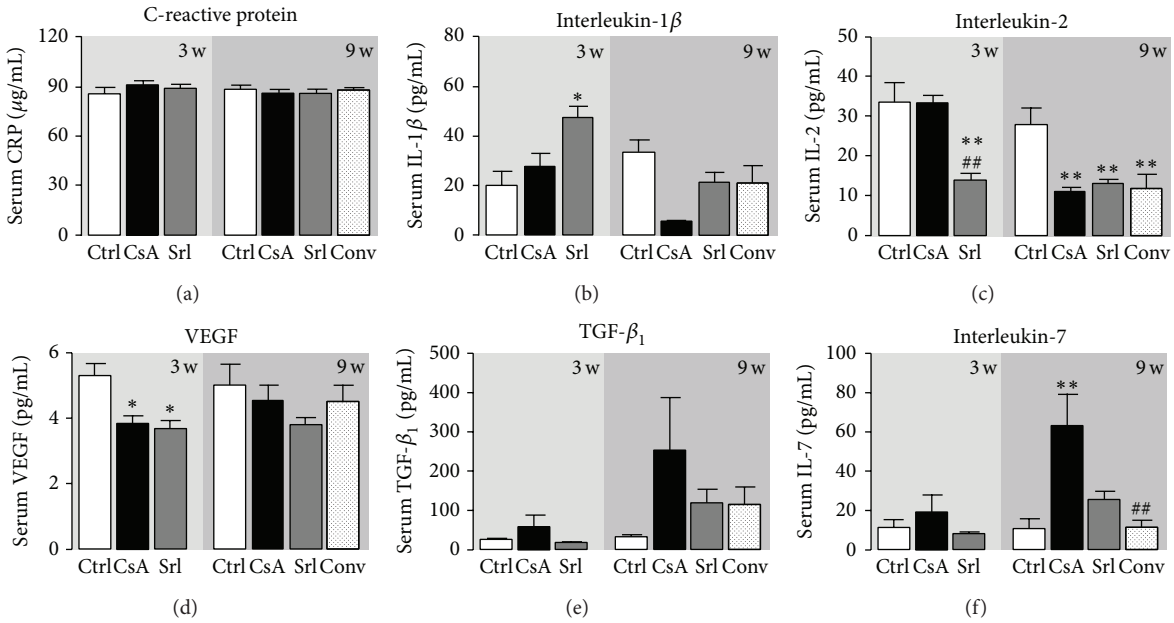

Figure 5: Serum markers of inflammation, proliferation, and angiogenesis. C-reactive protein (a), interleukin-1β (b), interleukin-2 (c), vascular endothelial growth factor (d), transforming growth factor beta-1 (e), and interleukin-7 (f). Values are mean ± SEM. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus cyclosporin A.

vascular congestion and hyperemia were not prevented when using SRL after CsA. Overall, we can conclude that total vascular scoring in the conversion group remained similar to the CsA group, but glomerular and tubular lesions scores were clearly reduced due to conversion to SRL. In addition, in the CsA-treated rats there was development of kidney fibrosis through collagen formation and deposition around vessels and tubules, together with bowman's capsules thickening. SRL, per se, or even after CsA treatment, was unable to present fibrosis or collagen deposition.

After 3 weeks of CsA exposure very slight changes on tissue structure were found, with absent or only mild lesions; however, after the long-term CsA exposure, significant glomerular, tubular, and vascular lesions were observed. In spite of that, at week 9 renal markers used in clinical practice (GFR, creatinine and BUN contents and clearances) appeared only modestly changed. Additionally, we observed an interesting variation of MDA clearance levels between 3 and 9 weeks of CsA treatment, when renal lesions were clearly noted. Knight et al. detected high MDA levels in urine of transplanted patients, but they were unable to explain their importance [35]. Our data suggests that MDA clearance could be a predictive marker of CsA-induced nephrotoxicity, as increased MDA clearance appears at the same time point as the first kidney lesions. Oxidative stress can promote the formation/release of a variety of vasoactive mediators [36] that can affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient, thus reducing the GFR. Moreover, the relationship between proteinuria and CsA-evoked nephrotoxicity is complex, limiting its power as an early marker [13]. Lipid peroxidation occurs as a result of multiunsaturated lipids reacting with oxidizing agents, promoting oxidative stress in the kidney structures. Urinary MDA reflects the presence of renal damage, which may be the cause or the consequence of lipid peroxidation, and the correlation between MDA clearance and kidney lesion grade could be a good strategy to identify early CsA-induced nephrotoxicity. The presence of slight or low grade lesions on the chronic SRL treatment and in the conversion protocol groups reinforces this idea, because no significance increase was found in MDA clearance for both groups.

The development of noninvasive biomarker that could diagnose renal dysfunction early and also monitor the response to therapy, as well as the ability to predict severity and outcome, would be very valuable. It is also important to recognize that changes in serum creatinine and BUN concentrations primarily reflect functional changes in filtration capacity and are not genuine injury markers [37]. During the last years, there has been an effort to identify better accurate biomarkers of acute CsA-induced nephrotoxicity. Gelatinase-associated lipocalin (NGAL) has been indicated as an acute marker of nephrotoxicity [38, 39]. NGAL in urine and plasma could have a 10,000-fold and 100-fold concentration rise, respectively, from normal levels in cases of renal injury. This could make NGAL a potentially very sensitive marker of different degrees of renal wound. However, according to our data, short- and long-term CsA treatments (clearly described as a nephrotoxic drug) were unable to promote increased serum and urine NGAL levels. Curiously, urine and serum samples presented linearity in the SRL group and a strong correlation in the conversion group. SRL is described in the literature as a less nephrotoxic agent than the calcineurin inhibitors, but one of the effects better described is the development of SRL-evoked proteinuria [40, 41]. The elevation in urine and serum NGAL levels in the SRL-treated rats in

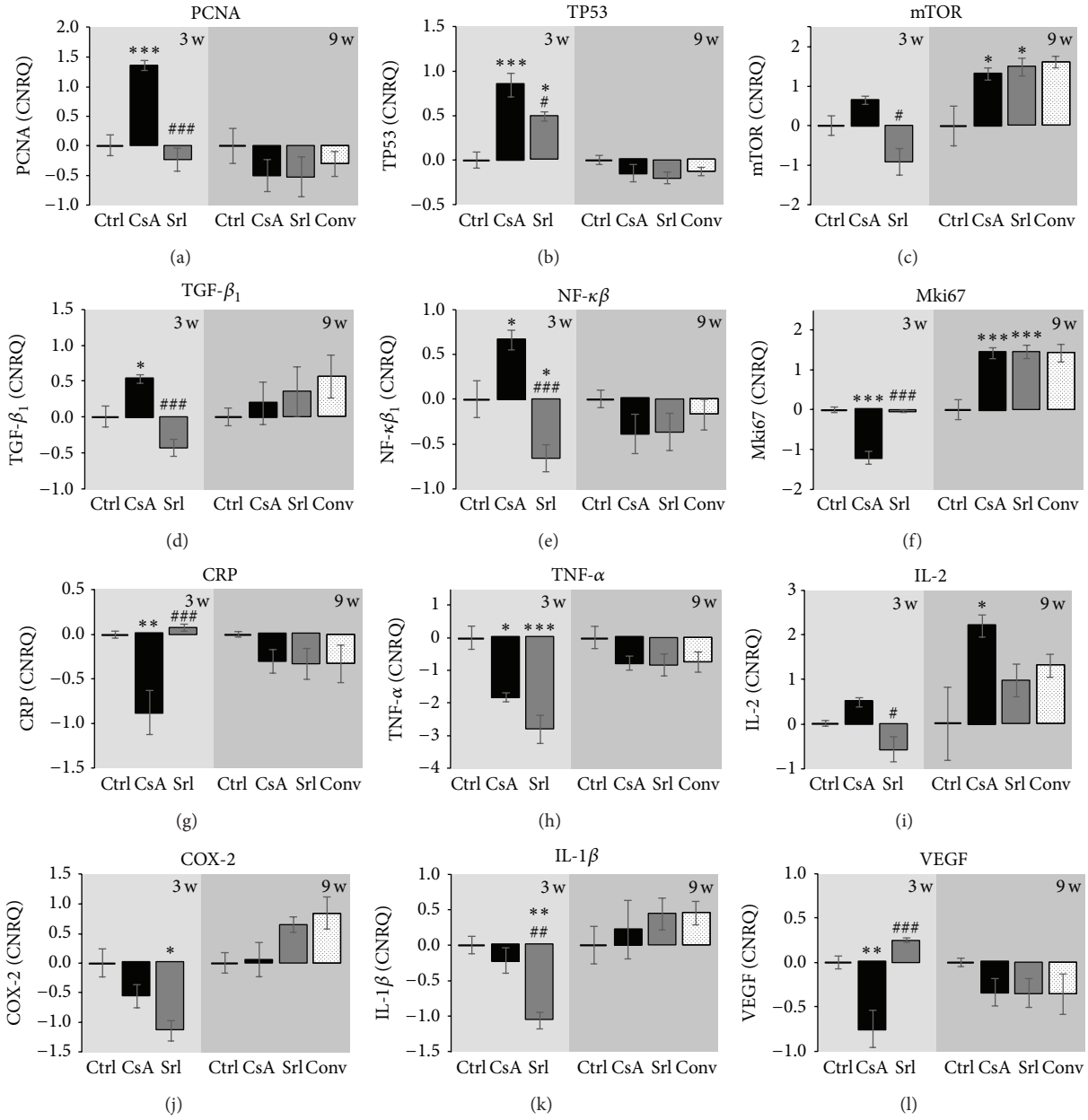

Figure 6: Kidney mRNA expression of proliferation, inflammation, and angiogenesis mediators. PCNA (a), TP53 (b), mTOR (c), TGF-β_1 (d), NF-κB (e), and Mki67 (f) as proliferation status markers; CRP (g), TNF-α (h), IL-2 (i), COX-2 (j), and IL-1β (k) as inflammation status markers and VEGF (l) as angiogenesis status marker. Values are mean of CNRQ (calibrated normalized relative quantities) of the control ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus cyclosporin A. COX-2, ciclooxigenase-2; CRP, C-reactive protein; IL-1β, interleukin-1 beta; IL-2, interleukin-2; MKi67, antigen identified by monoclonal antibody Ki-67; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; TGF-β_1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; TP53, tumor protein p53; VEGF, vascular epidermal growth factor.

our study could be related to the proteinuria appearance. Recently, a mouse mTOR knockout model revealed accumulation of autolysosomal vesicle in podocytes that potentiated proteinuria appearance [41] and reduced AKT activity, thus affecting podocyte cytoskeleton [42]. Moreover, concerning the tubular field, mTOR inhibition by using rapamycin has a role in the protein transport because it reduces tubular protein reabsorption that contributes to increasing urinary protein levels [43]. Furthermore, angiotensin II receptor blocker can counteract the effect of sirolimus, not only through hemodynamic changes but also partly by repairing the injury of podocytes [40].

In our study, a trend to increased serum and kidney TGF-β_1 was found in the CsA-treated rats, starting after just 3 weeks and aggravating with prolonged exposure, suggesting this factor as a putative good biomarker of nephrotoxicity

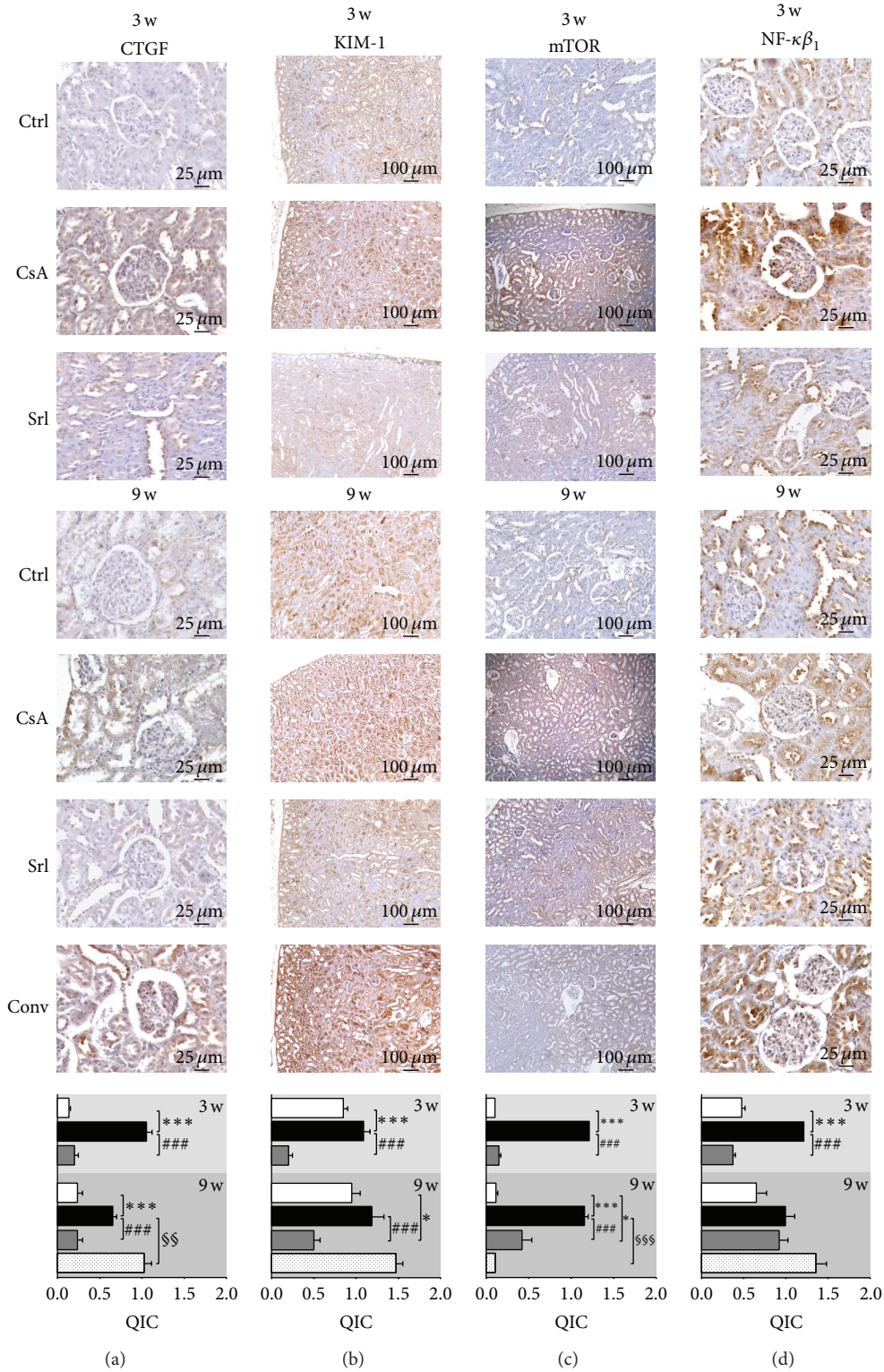

Figure 7: Kidney protein expression by immunohistochemistry. CTGF (a), KIM-1 (b), mTOR (c), and NF-κB_1 (d). Each figure is representative of the groups at week 3 (control, cyclosporin A, and sirolimus) and 4 groups at week 9 (control, cyclosporin A, sirolimus, and conversion). CTGF, connective tissue growth factor; KIM-1, kidney injury molecule-1; mTOR, mammalian target of rapamycin; NF-κB_1, nuclear factor kappa beta-1.

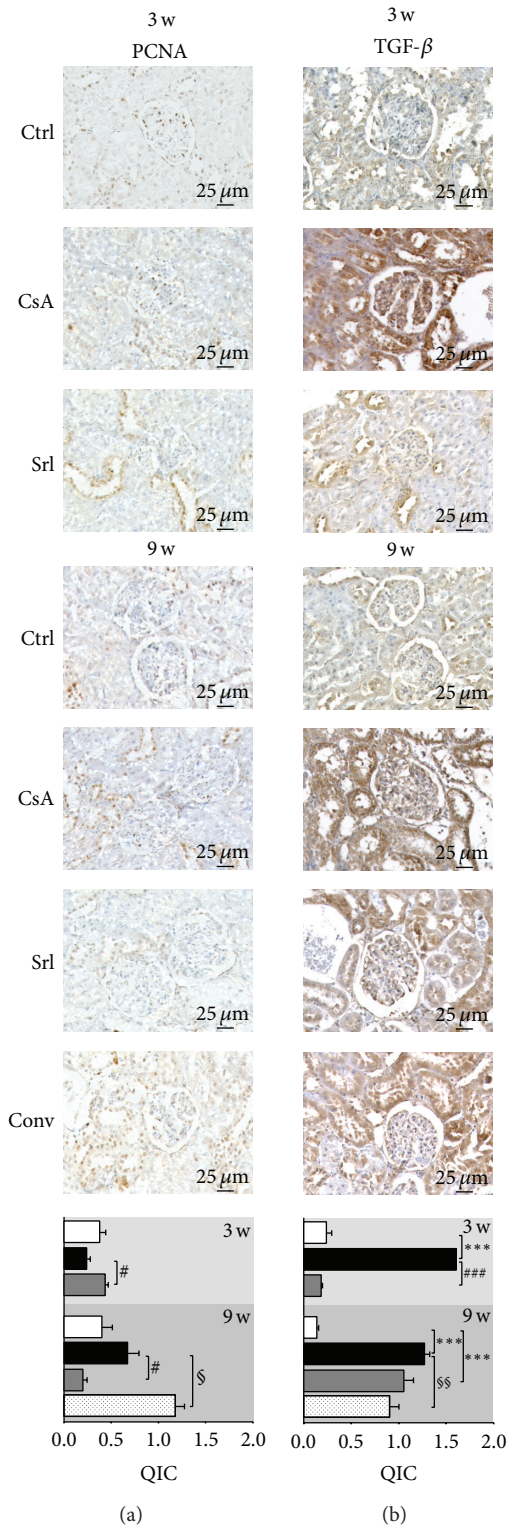

FIGURE 8: Kidney protein expression by immunohistochemistry. PCNA (a) and TGF-β_1 (b). Each figure is representative of the groups at week 3 (control, cyclosporin A, and sirolimus) and 4 groups at week 9 (control, cyclosporin A, sirolimus, and conversion). PCNA, proliferating cell nuclear antigen; TGF-β_1, transforming growth factor beta 1.

progression. Interestingly, similar pattern was encountered for serum IL-7 levels, showing significant correlation with serum TGF-β_1 contents in the short- and long-term treatments. IL-7 is produced constitutively by stromal cells and consumed by the available pool of resting T cells, all of which express the IL-7 receptor (IL-7R) at high levels except for $CD4^+$ $CD25^+$ regulatory T cells. Circulating IL-7 levels increase during periods of lymphopenia to maintain naïve T-cell homeostasis and support the thymic-independent peripheral expansion and maintenance of mature T cells [44] because they upregulate bcl-2 protein that has anti-apoptotic properties [45, 46]. CsA treatment decreases the immune system, specially T-cells number and activation, but alternatively the remaining immune cells can counterwork the immunologic depression by increasing IL-7 levels which could be correlated with the progression of chronic kidney disease in this study.

Changes in messenger RNA expression are considered to be one of the earliest events, which may occur in response to cellular and tissue damage; it has been speculated that these biomarkers might help to predict adverse effects before damage is indicated by the current gold standard markers (clinical chemistry and histopathology). Current theories point that renal damage is caused by nonimmunological factors, such as ischemia, which lead to activation of various proinflammatory and profibrotic mediators. A parallel concept of how CsA might induce renal injury was described by Li and Yang, suggesting that kidney damage involves activation of the innate immune response that causes NF-$\kappa\beta$ activation and induces dendritic cell maturation and T-lymphocyte infiltration into the graft, with both pathways ultimately resulting in interstitial inflammation and interstitial fibrosis that contributes to chronic nephropathy [47]. In agreement, our data confirm that CsA toxicity might start with increased NF-$\kappa\beta$ gene (RT-qPCR) and protein (immunohistochemistry) overexpression after 3 weeks of CsA treatment, an effect that is then downregulated with prolonged exposure. SRL treatment reduced mRNA levels, resulting in normal protein expression, when compared to control, both in short- and long-term treatments, indicating that this nuclear factor could have an important impact in the development of nephrotoxicity. At the end of the conversion protocol (CsA replaced by SRL), no difference was found in gene expression but protein overexpression remained in the tubulointerstitial region, most probably because of the previous CsA exposure. In our animal model, the short-term CsA treatment was mainly associated with upregulation of TGF-β_1 and PCNA in the kidney tissue, which has been identified as the key mediator of fibrosis and proliferation [47, 48]. However, these changes were accompanied by a putative compensatory response, since markers of inflammation (including COX2, TNF-α, and CRP), as well as of cellular proliferation (MKi67) and angiogenesis (VEGF), were downregulated, perhaps responsible for the attenuation of the cytotoxic effects of CsA in the short term. The overexpression of NF-$\kappa\beta$ and TP53 might be included in this compensatory response, since they inhibit mTOR [49]. Short-term SRL treatment revealed acute anti-inflammatory, antifibrotic, and antiproliferative properties, viewed by the downregulation of kidney mRNA levels of TNF-α, COX2, IL-1β, TGF-β_1, NF-$\kappa\beta$, and mTOR. Nevertheless, during prolonged CsA exposure, nephrotoxicity evolves, as viewed by the degree of increased histological lesions, which seems to be associated with other molecular pathways and mediators. In fact, there was a significant overexpression of MKi67, contrary to what was observed after the short-term treatment, suggesting a depletion of counter-regulatory responses, which was accompanied by a parallel increase in mTOR expression, a serine/threonine protein kinase, important in regulating cell growth, proliferation, motility, survival, protein synthesis, and transcription [48]. As Lieberthal and Levine demonstrated, mTOR plays an important role in mediating the process of regeneration and recovery, depending on the kidney damage extension [50]. Moreover, mTOR activity is low or absent in the normal kidney but increases markedly after acute kidney injury. In agreement, mTOR inhibition has been associated with amelioration of kidney fibrosis, glomerulosclerosis, and interstitial inflammation, having an important role in distinct renal diseases [50–52]. In our study, protein expression assessed by immunostaining revealed increased mTOR in the CsA-treated rats, which is in agreement with a previous study that suggested mTOR overexpression in CsA-treated rats, resulting in podocyte epithelial to mesenchymal transition leading to glomerular damage [53]. In addition, while normal kidney mTOR expression was found in the SRL-treated rats, there was an important decreased kidney expression in the conversion protocol group, which might explain the reduced lesions found when compared with the CsA monotherapy group.

In the last years, some toxicological studies showed hypothetical biomarkers that could predict acute nephropathy [32, 37, 54]. However, those studies were unable to assess if they could be viewed also as markers of chronic toxicity. Due to its functional reserve, minor effects on kidney function are too difficult to detect. Kidney injury molecule-1 (KIM-1) is a type 1 transmembrane protein expressed in the proximal tubules and further excreted in the urine; in the last years, KIM-1 has been pointed as a possible marker of renal injury in acute models. This factor has a role in proliferation and tissue repair [32, 55] because it confers phagocytic capacity to clear cell debris [56]. In our study, KIM-1 staining occurred in proximal tubule epithelial cells and might putatively be indicated as one of the most sensitive markers of tissue injury, in agreement with the previous suggestion of Rached et al. when studying nephrotoxin ochratoxin A [32]. In our study, after 3 weeks of CsA treatment, intense KIM-1 staining was found in the proximal tubules, but not after 9 weeks, when less stain intensity was found in all proximal tubules, suggesting that KIM-1 could be viewed as a putative good marker of acute CsA toxicity (without structural lesions), but not as a biomarker of chronic CsA treatment nephrotoxicity. KIM-1 is downexpressed in the kidneys of SRL-treated rats; however, when SRL was used to replace CsA (conversion group), a similar expression was found to that encountered for the CsA-treated rats after 3 weeks, suggesting that previous CsA exposure damaged some proximal tubules in an irreversible manner.

Connective tissue growth factor (CTGF) is a polypeptide implicated in the extracellular matrix synthesis that belongs to a profibrotic signalling (TGF-β_1 downstream modulator) and has been pointed as a possible biomarker of CsA-evoked damage. In our model, kidney CTGF expression increased after short- and long-term treatment with CsA, in agreement with the kidney overexpression of TGF-β_1 viewed by immunohistochemistry. After a longer CsA exposure the kidney expression of CTGF was slightly reduced, which might be explained by an increased urinary elimination, as previously suggested by O'Connell et al. in another experimental study [18]. SRL treatment per se does not promote any significant CTGF expression when compared with the control, in agreement with the absence of fibrosis or collagen deposition in the SRL-treated rats, as previously mentioned. However, SRL treatment after CsA therapy (Conversion protocol) was unable to restore basal levels of CTGF, suggesting that, once again, some of the lesions induced by CsA are maintained after the conversion for SRL.

In chronic kidney disease, rapamycin was able to slow the progression of renal fibrosis and delayed the onset of renal failure, through reduction of glomerular hypertrophy, decrease of proinflammatory and profibrotic cytokines production, and decline in interstitial inflammation [48]. As previously suggested, rapamycin is less fibrogenic than CsA [20], which is in agreement with the reduced kidney damage in the conversion protocol of our study. Our results reinforce the rationale for the early substitution of CsA by SRL, not only because longer CsA exposure is notoriously more deleterious, promoting structural kidney deterioration, but also because mTOR overexpression seems to be a feature of the chronic CsA exposure.

5. Conclusions

This experimental study demonstrated that CsA-induced nephrotoxicity is significantly aggravated over time and distinct mechanisms seem to underlie short- and long-term renal toxicity. The currently used clinical techniques and biomarkers, namely of biochemical impairment (such as serum and urine creatinine and BUN contents and clearance), if coupled with genetic and protein analysis in different samples, will bring more accuracy to early detect and follow up the appearance and development of nephrotoxicity. Conversion to SRL prevented CsA-induced renal damage evolution, which is better viewed by nontraditional, emergent biomarkers including serum TGF-β and IL-7, TBARs clearance, and kidney TGF-β and mTOR, while NGAL (serum versus urine) seems to be a feasible indicator of substitution to the mTOR inhibitor.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

José Sereno, Fedrerico Teixeira, and Flávio Reis conceived and designed the study protocol. José Sereno, Sara Nunes, Paulo Rodrigues-Santos, Helena Vala, Petronila Rocha-Pereira, João Fernandes, and Alice Santos-Silva performed experiments. José Sereno, Frederico Teixeira, and Flávio Reis analysed the data and prepared the paper. All authors have read and approved the paper.

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Research Article

Urokinase Gene 3′-UTR T/C Polymorphism Is Associated with Malignancy and ESRD in Idiopathic Membranous Nephropathy

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Idiopathic membranous nephropathy (MN) is one of the most common causes of nephrotic syndrome in adults, and 25% of MN patients proceed to ESRD. Urokinase plasminogen activator (uPA) may play an important role in reducing renal fibrosis. This study was conducted to clarify the relationship between uPA gene polymorphisms and clinical manifestations of MN. We recruited 91 biopsy-diagnosed MN patients and 105 healthy subjects. Genotyping of uPA gene 3′-UTR T/C polymorphism was performed by polymerase chain reaction methods. The genotype distribution had no effect on the development of MN. Thirteen patients (15.9%; $P = 0.008$) acquired malignancies and seventeen (20.7%; $P = 0.006$) patients progressed to ESRD with the C/C genotype, but no patients with the T/C genotype did. In conclusion, we demonstrated that the presence of the uPA gene 3′-UTR C/C genotype was associated with ESRD as well as acquired malignancies in MN patients. These findings should prompt specific considerations for the treatment of MN patients to maintain a balance between treating disease entities and protecting the immune system from cancers.

1. Introduction

End-stage renal disease (ESRD) is a major public health problem in Taiwan, which had the highest incidence and prevalence country in the world in 2007: 415 and 2,288 per one million people, respectively [1]. The annual mortality rate was 7.55 in 2007 [2] and the national prevalence of chronic kidney disease was 11.93% [3]. Renal disease was listed as the tenth leading cause of death at $13.2/10^5$ population in the 2009 Annual Report of the U.S. National Institutes of Health and the National Institute of Diabetes and Digestive and Kidney Diseases [1].

Membranous nephropathy (MN) is one of the common causes of ESRD in adult glomerulonephritis, also known to be the second most frequent cause of primary glomerulonephritis in Chinese people [4]. It is a prototype of an immune-mediated glomerular disease and is characterized by abundant, nonselective proteinuria and variable clinical course and prognosis [5–7]. The renal function and the course in MN are more strongly correlated with the degree of tubulointerstitial damage than with the extent of the glomerular lesion [8, 9], but the pathogenesis of the interstitial inflammation and fibrosis is unclear.

Genetic and environmental factors may contribute to disease progression and renal fibrosis in most types of renal disease. Identifying the genetic mechanism which may be related to the high incidence of MN is crucial for improving the current situation in Taiwan. In our previous study, we

demonstrated that the genotype distribution of plasminogen activator inhibitor type 1 (PAI-1) had no effect on the development of MN, and the 4G/4G genotype had significantly poorer creatinine clearance than the 4G/5G or 5G/5G genotypes in MN patients [10]. The frequency distribution of the G allele in genotype polymorphism of rs437168 (exon17) in the NPHS1 gene was significantly higher in MN patients than in controls, and a stratified analysis revealed a high disease progression in the AA genotype of rs401824 (5′UTR) and GG genotype of rs437168 (exon17) patients who were associated with a low rate of remission [11].

Urokinase plasminogen activator (uPA), a plasminogen activator synthesized by the kidney and other cells that triggers the cleavage of plasminogen to plasmin and hence induces fibrinolysis, may play an important role in reducing renal fibrinosis [12, 13]. Urinary uPA was significantly lower in patients with intraglomerular fibrin deposition than in patients without fibrin deposition. The decrease of urinary uPA levels and diminution of isolated intraglomerular plasminogen activator activity contribute to the progression of primary glomerular diseases [14]. UTRs are known to play crucial roles in the posttranscriptional regulation of gene expression, including modulation of the transport of mRNAs out of the nucleus and of translation efficiency, subcellular localization, and stability [15–17]. The urokinase gene 3′-UTR "T" allele is highly associated with calcium stone disease [18] and oral cancer [19]. The present study was therefore conducted to investigate the frequency distribution of urokinase gene 3′-UTR gene polymorphism associated with the risk of MN patients and healthy individuals in Taiwan and to explore the correlation of the clinical manifestation in different gene polymorphisms.

2. Methods

2.1. Study Population. We recruited a cohort of 92 biopsy-diagnosed MN patients and 105 healthy subjects, who served as controls during January 2000–December 2002. The follow-up period of MN patients was from their renal biopsy date to June 2011. Patients who had secondary MN with malignancy, chronic infectious diseases (including infections with hepatitis B and hepatitis C viruses), lupus nephritis, or drug-induced diseases were excluded from this study. The patient characteristics and medical records were reviewed, including demographic variables, clinical and laboratory data for the disease courses, vascular events, and treatment regimens as well as their responses. Informed consent was obtained from all participating individuals and the study protocol complied with the ethical guidelines of our hospital (VGHTC IRB number C08159).

The selection of treatment modality, either supportive or aggressive with immunosuppressants, was based on the treating physician's decision. The supportive therapy usually included diuretics, angiotensin converting enzyme inhibitors (ACEIs), and/or angiotensin II receptor blockers (ARBs), depending on the patient's symptoms. The immunosuppressive therapies included any of the following regimens: (1) prednisolone 1 mg/kg/day alone, (2) a six-month course of corticosteroids alternating with chlorambucil at a dose of 0.2 mg/kg/day every other month [20] or cyclophosphamide 1.5–2.0 mg/kg/day, or (3) cyclosporine A (CyA, Novartis Inc., Basel, Switzerland) 3–5 mg/kg/day with or without prednisolone.

2.2. Responses and Outcomes. The responses to therapy were defined as follows: (1) no response, (2) partial remission: a proteinuria reduction of more than 50% or final proteinuria between 0.2 and 2.0 g/day, and (3) complete remission: proteinuria less than 0.2 g/day. The progression of renal disease was defined as doubling baseline serum creatinine (Cr) values or ESRD. ESRD was defined as required renal replacement therapy.

2.3. Renal Biopsy Review. The histological staging was based on histological lesion, including glomerular lesion [16], tubulointerstitial lesion, focal glomerulosclerosis [17], and fibrointimal lesion. The renal biopsy specimens were reviewed by a nephropathologist, who was blinded to patients' clinical history, renal function, and urokinase gene 3′-UTR T/C polymorphism. A semiquantitative scoring system was adopted using a scale of 0 (none), 1 (mild: less than 25%), 2 (moderate: 25 to 50%), and 3 (severe: more than 50%) for the assessment of tubulointerstitial change and glomerular sclerosis/obsolescence under light microscopy. Staging of disease was also determined according to findings using electron microscopy.

2.4. Determination of Urokinase Gene 3′-UTR T/C Polymorphism. PCRs were carried out to a total volume of 50 μL containing genomic DNA, 2 to 6 pmol of each primer, 1X Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 U AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). The primer for the urokinase gene 4065 T/C polymorphism was designed as 5′-CCGCAGTCACACCA AGGAAGAG-3′ and 5′-GCCTGAGGGTAAAGCTA TTGTCGTGCAC-3′, according to the published data from Medline (STS Accession number G27040). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). The cycling condition for urokinase gene 3′-UTR T/C polymorphism was set as follows: one cycle at 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, and one final cycle of extension at 72°C for 7 minutes.

The PCR product of 210-bp was mixed with 2 U *ApaL* I (New England Biolabs, Beverly, MA) and the reaction buffer, according to the manufacturer's instructions. The restriction site was designed to be located at the allele of 3′-UTR (T) to form a digestible site. Two fragments of 185-bp and 25-bp will be present if the product is digestible. The reaction was incubated for 3 hours at 37°C. Then, 10 μL of the product was loaded into 3% agarose gel plates containing ethidium bromide for electrophoresis. The polymorphism 343cbe was divided into three groups: digestible (T/T homozygote), indigestible (C/C homozygote), and C/T heterozygote.

2.5. Urine uPA Measurement. A 20 mL of urine was collected into clean 15 mL tubes for immediate freezing and storage in −20°C freezers. Urine uPA levels were determined by using the suPARnostic ELISA kit (ViroGates, Copenhagen, Denmark). The assay comprised plates precoated with a catching monoclonal antibody for loading the sample and an HRP-labelled detection monoclonal antibody that was added to the sample dilution buffer. Briefly, 25 μL of urine sample was mixed with 225 μL of dilution buffer added to the plates and incubated for one hour. After washing the plates, 50 μL of substrate was added for 20 min and the reaction was stopped with 50 μL 0.5 M H_2SO_4. Plates were measured at 450 nm in a spectrophotometer.

2.6. Statistical Analysis. Continuous variables are expressed as mean and standard deviation. Urokinase gene 3′-UTR T/C genotype and allele frequencies between MN and normal controls were compared using Chi-square analysis. The relationships between urokinase gene 3′-UTR T/C genotypes, patient characteristics, and histology were also compared by Chi-square test. Differences in various clinical parameters among T/T, T/C, and C/C genotypes were compared using the analysis of variance (ANOVA).

Kaplan-Meier survival analysis was used to determine kidney survival and patient survival. The survival rate among different urokinase gene 3′-UTR T/C genotypes was compared by means of a two-sided Log-rank test. Differences were considered statistically significant when $P < 0.05$. All analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Population Study. The distribution of urokinase gene 3′-UTR (SNP4065) polymorphism among MN and healthy control subjects was not significantly different, as shown in Table 1. Neither allelic frequency nor carriage rates of urokinase gene 3′-UTR polymorphism were observed in MN or healthy control subjects (Table 1). No MN patients carried the T/T homozygote. Only 9 (13%) MN patients carried the T/C genotype, whereas 82 (90%) MN patients carried the C/C genotype, which was similar to the percentage for healthy controls (Table 1).

3.2. Relationship between Urokinase Gene 3′-UTR T/C Polymorphisms and Clinical Features of MN. The minimal follow-up duration was 1 year, except for four patients who died of pneumonia; two had respiratory failure (0.9 months and 1.4 months), one had septic shock (2.2 months), and one had subdural hemorrhage with urosepsis (1.5 months), respectively. The clinical features of the 2 genotypes of urokinase gene 3′-UTR polymorphism are shown in Table 2. There were no differences in gender distribution, age at onset, duration of follow-up, body mass index, mean blood pressure (MBP), hematuria, or proteinuria. The baseline laboratory data revealed similarities between the serum creatinine level (Cr), creatinine clearance (CCr) level, and daily urinary protein excretion (DUP) in Table 2. After a mean duration of 9.5 ± 6.0 years' follow-up, the last Cr measurement in patients with the C/C genotype (3.3 ± 4.3 mg/dL) was higher than that in patients with the T/C genotype (1.2 ± 0.4 mg/dL), but the difference did not reach statistical significance. The last CCr measurement (52.1 ± 39.2 mL/min) in patients with the C/C genotype was lower than that in patients with the T/C genotype (76.4 ± 26.7 mL/min), though without statistically significance ($P = 0.075$) in Table 2. The pathological features also disclosed no difference between the MN grade, percentage of glomerulosclerosis, tubulointerstitial fibrosis score, or fibrointimal atherosclerosis score between the two genotypes in MN patients (data not shown).

Table 1: Distribution of 3′-UTR gene polymorphism (SNP4065) of urokinase gene among idiopathic membranous nephropathy and healthy control subjects.

Genotype	MN ($N = 91$)	Control ($N = 105$)	P value
Male gender	50 (54)	61 (58)	0.325[a]
Age (yrs)	57.8 ± 16.4	49.8 ± 14.4	0.000[b]
Genotype of urokinase gene			
C/C	82 (90%)	90 (86%)	0.237[c]
C/T	9 (10%)	15 (14%)	
T/T	0 (0%)	0 (0%)	
Allelic frequency			
C	173 (95)	195 (93)	0.238[a]
T	9 (5)	15 (7)	
Allelic carriage			
C	91 (100)	105 (100)	0.318[a]
T	9 (10)	15 (14)	

Number in the parenthesis is percentage.
[a]Yates' correction of contingency.
[b]Mann–Whitney U test.
[c]Chi-square test.
Patient age, which was used as a covariate to adjust the genotype in the study population by multinomial logistic regression, as not significantly different between control and MN groups.

3.3. Relationship between Urokinase Gene 3′-UTR T/C Polymorphisms and Outcomes in MN. There is no consensus on the standard treatment for patients with idiopathic membranous nephropathy. Generally, high-risk patients are treated with immunosuppressive therapy, such as steroids in combination with chlorambucil or cyclophosphamide, and cyclosporine. Although our patients received the best available treatment regimens, only 42 (51.2%) patients with the C/C genotype and 6 (66.7%) patients with the T/C genotype achieved complete remission. Forty-seven (57.3%) patients with the C/C genotype and 3 (33.3%) patients with the T/C genotype had disease progression. MN progressed to ESRD in 17 (20.7%) patients with the C/C genotype, but no MN patients with the T/C genotype had progression to ESRD ($P = 0.006$, Table 3). These results indicated that MN patients who carry the C/C genotype in the 3′-UTR urokinase gene have poor response to treatment modalities and aggravated renal function leading to ESRD. The urine uPA levels were

Table 2: The clinical characteristics and urokinase gene 3′-UTR gene polymorphism and its activity.

	C/C ($n = 82$)	C/T ($n = 9$)	P value
Male gender (%)	42 (51.8%)	7 (77.8%)	0.121
Age of onset (yrs)	52.5 ± 15.7	51.4 ± 25.2	0.847
Age of biopsy (yrs)	58.2 ± 15.4	53.9 ± 25.0	0.463
Follow-up period (yrs)	9.6 ± 6.0	7.8 ± 5.8	0.390
BMI (Kg/M^2)	24.7 ± 3.6	24.0 ± 3.3	0.557
MBP (mmHg)	98.6 ± 12.2	101.5 ± 24.2	0.553
Albumin (mg/dL)	2.5 ± 0.6	2.7 ± 0.5	0.338
Cholesterol (mg/dL)	347.7 ± 137.3	298.9 ± 113.3	0.307
Triglyceride (mg/dL)	236.3 ± 165.0	161.2 ± 119.5	0.189
$Cr_{initial}$ (mg/dL)	1.3 ± 1.1	1.4 ± 1.1	0.788
$DUP_{initial}$ (g/day)	7.0 ± 8.2	7.1 ± 4.7	0.974
$CCr_{initial}$ (mL/min)	87.6 ± 41.2	81.0 ± 45.6	0.655
PT (second)	11.2 ± 0.8	11.6 ± 0.8	0.290
aPTT (second)	27.0 ± 5.2	30.0 ± 3.2	0.144
Cr_{final} (mg/dL)	3.3 ± 4.3	1.2 ± 0.4	0.150
DUP_{final} (g/day)	2.6 ± 3.5	1.1 ± 1.3	0.197
CCr_{final} (mL/min)	52.1 ± 39.2	76.4 ± 26.7	0.075
Proteinuria ≧ 3.5 g/day	28 (34.1%)	3 (33.3%)	0.838
Hematuria	46 (56.1%)	4 (44.4%)	0.374
Lower leg edema	69 (84.1%)	7 (77.8%)	0.451
Urine uPA (ng/mL)	6.17 ± 4.68	6.06 ± 5.37	0.962

BMI: body mass index; MBP: mean blood pressure; DUP: daily urinary protein excretion; CCr: creatinine clearance. All data are presented as mean ± SD; urine uPA: urine urokinase plasminogen activator. There were only 28 urine samples collected from patients with C/C ($n = 22$) and C/T genotypes ($n = 6$) of urokinase gene 3′-UTR gene polymorphism for the functional study of urine uPA.

Table 3: 3′-UTR of urokinase gene polymorphism and clinical outcome.

	C/C ($n = 82$)	C/T ($n = 9$)	P value
Cardiovascular events	17 (20.7)	2 (22.2)	0.917
Malignancy	13 (15.9)	0 (0.0)	0.008*
Complete remission	42 (51.2)	6 (66.7)	0.641
Disease progression	47 (57.3)	3 (33.3)	0.154
ESRD	17 (20.7)	0 (0.0)	0.006*

Distribution was analyzed by Chi-square test.
*Measured by Kendall's Tau-b significant P value < 0.05.

compared to strengthen the functional study of uPA genes 3′-UTR C/C ($n = 22$) and T/C ($n = 6$) genotypes, without significant difference between two genotypes in Table 2.

The cardiovascular events were similar in both genotypes. Thirteen (15.9%) patients with the C/C genotype developed malignancies during follow-up, whereas no patients with the T/C genotype did ($P = 0.008$, Table 3). Most of the patients received immunosuppressive therapies, including prednisolone (12), a combination of prednisolone and cytotoxic agents (5) and CsA (3) for MN, and one received ACEI therapy only. The median period of onset of malignant neoplasms was 9.9 ± 6.1 years. The pattern of malignancy (Table 4) was different from that of the three leading types of cancer in Taiwan: lung cancer, hepatoma, and breast cancer. The MN patients with malignancy received surgical intervention in 8 (61%) and chemotherapy in 3 (23%). Five of them were mortality with function kidney, and four patients renal failure before death.

3.4. Survival Analysis of MN Patients with Different 3′-UTR Genotypes of the Urokinase Gene. Figure 1 shows the Kaplan-Meier curves for renal survival and patient survival according to the distributions of the 3′-UTR genotypes of the urokinase gene. Although there was a trend towards better renal survival and patient survival in T/C patients (100%), this difference was not significant due to the small sample size.

4. Discussion

The current study showed that urokinase gene 3′-UTR polymorphism was not correlated with the development of MN. However, we clearly demonstrated that the presence of the C/C allele in MN patients was associated with ESRD and possibly with the occurrence of malignant neoplasms. These data strongly suggest poor prognosis for MN patients with the C/C genotype. Although renal survival and patient survival rates were not significantly different, a trend towards improved survival was found in patients with the T/C genotype. Because MN is an insidious disease with a protracted clinical course, the follow-up duration of the current study may be too short and the sample size may be too small to detect a meaningful difference in survival between the two subgroups.

The exact mechanism by which the C/C genotype exerts its detrimental effect is not fully understood. Our previous study demonstrated that the presence of the 4G allele was associated with renal deterioration in MN patients [9]. The expression of PAI-1, an inhibitor of uPA and tissue plasminogen activator (tPA), in injured kidney is associated with enhanced recruitment of interstitial macrophages and myofibroblasts not only from increased matrix protein synthesis but also from decreased degradation by connective tissue proteases [21, 22]. uPA, which is copiously produced by proximal and distal tubules, is a logical source of endogenous renal antifibrotic activity. However, uPA is normally excreted apically into the urinary space, and whether significant interstitial delivery occurs when the kidney is damaged is unknown [22]. In a primary glomerulopathy study, the urinary uPA levels were significantly reduced by intraglomerular fibrin deposition, which suggests that a decrease in urinary uPA levels and diminution of isolated intraglomerular plasminogen activator activity contribute to disease progression [14].

In this study, we demonstrated that MN patients with the C/C genotype in the 3′-UTR urokinase gene had poor response to treatment modalities and aggravated renal function leading to ESRD. The interaction of RNA-binding proteins with 5′- or 3′-untranslated regions (UTRs) of mRNA is the translational control mechanism. Protein-mediated interactions between transcript termini result in the formation of

Table 4: The malignant neoplasm in C/C genotype of 3′-UTR of urokinase gene polymorphism in MN patients and their presentation.

Malignancy	Malignancy duration (yrs)			Management
	Diagnosis	Renal failure	Mortality	
Lymphoma (3)				
Diffuse large B cell lymphoma, brain	25.0	Before	2.6/death	Chemotherapy
Malignant lymphoma, mixed large and small cleaved cell, B phenotype, with bone marrow involvement, stage IV	5.5	1.7/function	1.7/death	Chemotherapy
Angioimmunoblastic T-cell lymphoma	5.5	0.1/function	0.1/death	No
Skin cancer (2)				
Basal cell carcinoma, left nasal base and medial canthus	15.5	1.7/function	1.7/death	Incision
Squamous cell carcinoma, skull	2.9	2.2/failure	3/death	Incision
GI tract malignancy (3)				
Adenocarcinoma, stomach	15.0	0.1/function	0.1/death	No
Adenocarcinoma, stomach	7.6	2.5/function	2.5/survival	Subtotal gastrectomy
Adenocarcinoma, rectum	6.2	7.3/function	7.3/survival	polypectomy
GU tract malignancy (2)				
Prostate adenocarcinoma	9.5	Before	5.9/death	Radical prostatectomy
Renal cell carcinoma, left kidney	3.2	4.3/failure	5.2/death	Left nephrectomy
Gynecology malignancy (2)				
Squamous cell carcinoma, cervix	10.9	1.2/function	1.2/survival	Vaginal hysterectomy
Squamous cell carcinoma, cervix	10.6	Before	2.3/survival	Vaginal hysterectomy
Respiratory tract (1)				
Adenocarcinoma, LLL, lung	11.9	0.6/function	0.6/death	Chemotherapy

Before: renal failure before diagnosis of malignancy.

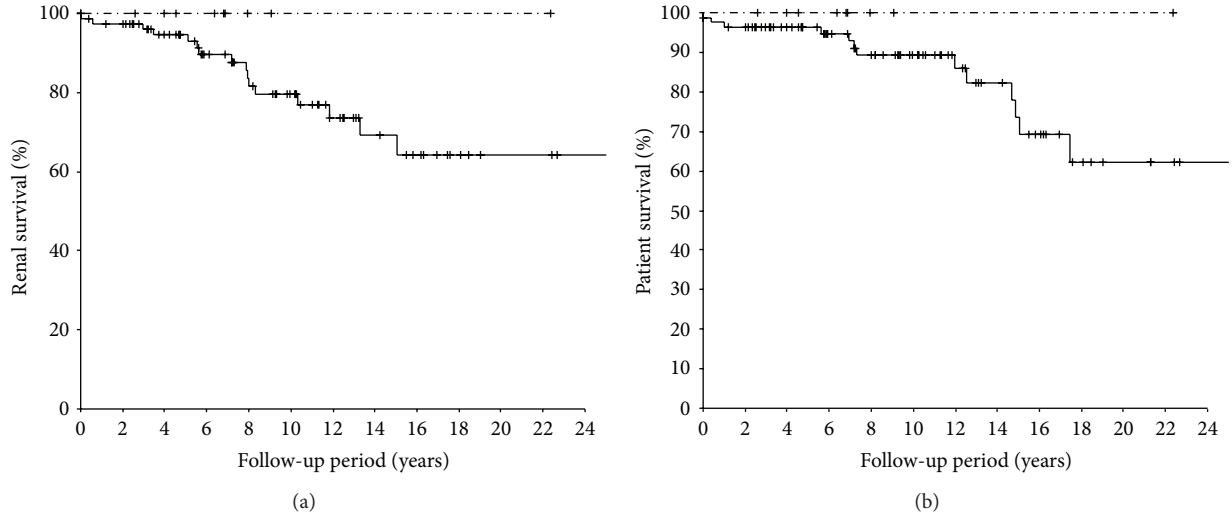

Figure 1: (a) Kaplan-Meier plot of renal survival stratified by urokinase gene 3′-UTR gene polymorphism (C/C: — T/C: ·-·) in membranous nephropathy. Mean renal survival was 19.8, 95% confidence interval 17.1–22.4. (b) Kaplan-Meier plot of patient survival stratified by urokinase gene 3′-UTR gene polymorphism (C/C: — T/C: ·-·) in membranous nephropathy. Mean survival was 20.6 yrs. 95% confidence interval: 18.2–23.1 in C/C group.

an RNA loop in all species, which is thought to increase translational efficiency and to permit regulation by new mechanisms, particularly 3′-UTR-mediated translational control [15–17, 23]. In many of the previous studies, the urokinase gene 3′-UTR T allele was associated with higher incidence of calcium oxalate stone disease [18], rheumatoid arthritis [24], oral cancer [19], prostate cancer [25], bladder cancer [26], and Alzheimer's disease [27], but some other studies indicated that the T allele is not associated with calcium oxalate nephrolithiasis [28] or bronchopulmonary dysplasia

in ventilated preterm infants [29]. To our knowledge, there was no correlation of uPA activity with urokinase gene 3′-UTR genotypes in previous studies, and we did not find such correlation in this study. It was recently reported that uPA activity may have organ-specific effects during fibrotic response, and various experimental interventions with high uPA could decrease fibrosis in the lungs and liver and increase fibrosis in the heart in mice [30, 31]. In a mouse UUO model, there was no significant difference in the degree of renal fibrosis between uPA wild-type and knockout mice [32], but uPA may interact with several distinct cellular receptors, including some that promote and others that inhibit renal fibrosis [30, 33]. The most important cellular receptor is the high-affinity receptor for urokinase (uPAR). During renal insult, the inducible uPAR enhanced expression of some renal tubules, inflammatory cells, and interstitial myofibroblasts [34]. Studies on uPAR genetically deficient mice have demonstrated that uPAR plays a protective role during the kidney's response to injury [34–36]. The mechanism of renal fibrosis is complex and not yet clear in humans. Therefore, further investigation of the relationship between uPA activity and uPAR in serum and pathology is needed.

In this study, 13 MN patients (15.9%) with the C/C genotype acquired malignancies during long-term follow-up. uPA has a proteolytic effect on degradation of the extracellular matrix (ECM), which allows malignant cells to invade locally and eventually spread distally. High levels of uPA components have been shown to predict adverse outcome in different types of malignancy and are consistent with cancer progression [36]. The present study demonstrated that a high rate of malignancy was associated with the urokinase gene 3′-UTR C/C genotype in MN, but the relationship needs further clarification before definitive conclusions can be made. Our 13 MN patients received strong immunosuppressive agents for several years to counteract the poor response of proteinuria; thus, most of their cancers were those associated with known or suspected viral causes, such as lymphoma, cervical cancer, and skin cancer [37]. The results were similar to the results for organ transplant recipients, which suggest that immunity was oversuppressed in our strategy to manage resistant MN. Patients with malignancies are frequently exposed to risk for renal injuries associated with disease-related or iatrogenic causes. Nephrotoxicity is a potential adverse effect of anticancer agents (e.g., gemcitabine and cisplatin) especially in patients with vulnerable chronic kidney disease (CKD) like MN. Increased understanding of the mechanism of renal injury by these agents, it is important to avoid adverse effects on CKD patients by adjusting their dosage [36, 37].

Although our study had the limitation of small sample size, there were no significant differences among the initial clinical characteristics and pathological features, the modes of treatment, or the follow-up duration in the two genotypes. On the other hand, the urokinase gene 3′-UTR T allele is rare in Taiwan [13, 14]. The small sample population and rare allele frequency might have a reduced chance of detecting a true effect and also could reduce the statistically significant result that reflects a true effect.

In conclusion, we demonstrated that the presence of the urokinase gene 3′-UTR C/C genotype was associated with ESRD as well as acquired malignancies in MN patients. These findings should prompt specific considerations for the treatment of MN patients to maintain a balance between treating disease entities and protecting the immune system from cancers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Effects of Single Pill-Based Combination Therapy of Amlodipine and Atorvastatin on Within-Visit Blood Pressure Variability and Parameters of Renal and Vascular Function in Hypertensive Patients with Chronic Kidney Disease

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Both strict blood pressure (BP) control and improvements in BP profile such as BP variability are important for suppression of renal deterioration and cardiovascular complication in hypertension and chronic kidney disease (CKD). In the present study, we examined the beneficial effects of the single pill-based combination therapy of amlodipine and atorvastatin on achievement of the target BP and clinic BP profile, as well as markers of vascular and renal damages in twenty hypertensive CKD patients. The combination therapy with amlodipine and atorvastatin for 16 weeks significantly decreased clinic BP, and achievement of target BP control was attained in an average of 45% after the combination therapy in spite of the presence of no achievement at baseline. In addition, the combination therapy significantly decreased the within-visit BP variability. With respect to the effects on renal damage markers, combination therapy with amlodipine and atorvastatin for 16 weeks significantly decreased albuminuria (urine albumin-to-creatinine ratio, 1034 ± 1480 versus 733 ± 1218 mg/g-Cr, $P < 0.05$) without decline in estimated glomerular filtration rate. Concerning parameters of vascular function, the combination therapy significantly improved both brachial-ankle pulse wave velocity (baPWV) and central systolic BP (cSBP) (baPWV, 1903 ± 353 versus 1786 ± 382 cm/s, $P < 0.05$; cSBP, 148 ± 19 versus 129 ± 23 mmHg, $P < 0.01$). Collectively, these results suggest that the combination therapy with amlodipine and atorvastatin may exert additional beneficial effects on renal and vascular damages as well as BP profile in addition to BP lowering in hypertension with CKD.

1. Introduction

Chronic kidney disease (CKD) patients are reportedly increasing in number, and cardiovascular complications are the most common cause of death in these patients. Thus, it would be a considerable advance in the management of this condition to elucidate the mechanisms involved in the renal deterioration and the cardiovascular events associated with hypertension complicated by CKD and to identify therapeutic approaches to treat them. Accumulated results of clinical trials also showed that strict control of blood pressure (BP) is essential to prevent target organ damage and to reduce cardiovascular mortality in hypertensive CKD patients [1, 2]. The dihydropyridine calcium channel blocker (CCB) is one of the first-line antihypertensive drugs for most patients with hypertension and is known to exert an efficient BP lowering effect and a strong inhibitory effect on cardiovascular events [3, 4].

In addition, therapies that lower lipid levels also slow the progression of atherosclerosis and reduce morbidity and

mortality in patients with hypertension or atherosclerotic disease. The Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) demonstrated an additive benefit of combined antihypertensive and lipid-lowering therapy on the prevention of cardiovascular complication in high-risk hypertension [5]. Vascular dysfunction, with associated changes in endothelial function and vascular structure, is a risk factor for cardiovascular events through its contribution to the development of atherosclerotic vascular disease. Previous investigations demonstrated improved vascular function and arterial compliance with statins and several antihypertensive drugs such as renin-angiotensin system inhibitors and CCB [6–8]. Both amlodipine and atorvastatin have independently been noted to exert favorable effects on arterial compliance and endothelial dysfunction [8, 9]. This study aimed to examine the beneficial effects of single pill-based combination therapy with amlodipine and atorvastatin on clinic BP profile including within-visit BP variability, a recently emerging marker of linking between kidney and vasculature, and parameters of vascular and renal function in Japanese hypertensive CKD patients who did not achieve the target BP level according to the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH2009) [10].

2. Materials and Methods

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committees of Yokohama City University Hospital (UMIN 000009045; http://www.umin.ac.jp/ctr/). All of the patients provided written informed consent prior to the start of the study.

2.1. Study Participants and Design. Hypertensive patients with CKD who have already been treated with antihypertensive therapy were eligible for the study if they could not achieve the BP goal (clinic systolic BP ≥ 130 mmHg and/or diastolic BP ≥ 80 mmHg), and their low-density lipoprotein (LDL) cholesterol levels were 100 mg/dL or more. CKD was diagnosed by the presence for more than 3 months of albuminuria (urine albumin-to-creatinine ratio, UACR ≥ 30 mg/g-Cr), proteinuria (urine protein-to-creatinine ratio, UPCR ≥ 0.15 g/g-Cr), or estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2. We calculated the eGFR using a revised equation for the Japanese population: eGFR (mL/min/1.73 m^2) = 194 × serum creatinine$^{-1.094}$ × age$^{-0.287}$ × 0.739 (if female) [11]. The exclusion criteria included CKD patients of G5 category, patients who were 19 years old or younger, women who were nursing or pregnant, clinically significant heart disease, stroke, renal artery stenosis, hepatic dysfunction, and known hypersensitivity to any ingredient in the study medications.

After the run-in period, eligible patients were given a single pill of amlodipine/atorvastatin tablet (CADUET, amlodipine besylate/atorvastatin calcium; 2.5/5, 2.5/10, 5/5, and 5/10 mg) for 16 weeks. The starting dose of the single amlodipine/atorvastatin tablet was determined according to each physician's choice, and it was titrated up as needed to achieve the BP and lipid goal (clinic systolic BP < 130 mmHg, diastolic BP < 80 mmHg, and LDL-cholesterol < 100 mg/dL). If patients had already been treated with another calcium channel blocker or statin before the start of the study, they were switched to the single amlodipine/atorvastatin tablet of same dose during the run-in period. If the BP and lipid goal could not be achieved by maximum doses of amlodipine and atorvastatin (10 mg/day and 20 mg/day), another antihypertensive drug and lipid-lowering drug were considered to be added. The doses of other drugs such as oral glucose lowering agents and anticoagulant agents were not changed during the treatment period.

2.2. Clinic BP and Within-Visit BP Variability. The clinic BP was measured in sitting position using a calibrated standard mercury sphygmomanometer and the recommended cuff size [12]. Three measurements were taken at 1-minute interval, and their average was regarded as the clinic BP. Within-visit BP variability, which has been reported to be associated with the risk of stroke and cardiovascular risk factors [13, 14], is defined as the within-patient standard deviation (SD) of three measurements of clinic systolic and diastolic BP (within-visit BP variability, SD) and those divided by the each mean clinic BP (within-visit BP variability, CV%).

2.3. Central Hemodynamics. The central systolic blood pressure (cSBP) and augmentation index (AI) were measured using an HEM-9000AI (Omron Healthcare, Kyoto, Japan) with an automatic tonometry probe wrapped onto the wrist to record radial waveforms, which were calibrated against the contralateral arm cuff brachial BP taken immediately after tonometry. An algorithm based on a linear regression model was then applied to estimate the cSBP from the "late systolic shoulder" (pSBP2) of the radial pulse waveform, which has been shown to closely agree with the cSBP [15–18]. This device uses the maxima of the "multidimensional derivatives" on the recorded pressure waveforms to detect the first and second inflection points corresponding to the early and late systolic (pSBP2) pressure readings.

2.4. Brachial-Ankle Pulse Wave Velocity (baPWV). The baPWV values were determined by a PP analyzer (model: BP-203RPE2; Omron Healthcare, Kyoto, Japan), as described previously [19–21]. The baPWV values obtained by this method are reported to be significantly correlated with the aortic PWV determined by the catheter method [22].

2.5. Laboratory Measurements. Blood and urine sampling were performed in fasted state at baseline and after a period of 16-week treatment, respectively. All parameters were determined by routine methods in the Department of Clinical Chemistry, Yokohama City University School Hospital.

2.6. Statistical Analysis. All data were presented as the mean ± SD or as a percentage. For the statistical analysis of the difference between baseline and 16 weeks of treatment, analysis of variance was compared by a paired comparison

Table 1: Baseline characteristics.

Variables	Mean ± SD or %
Age (y)	67.7 ± 12.4
Sex (male/female)	14/6
Body mass index (kg/m^2)	26.8 ± 4.9
Alcohol (%)	40
Smoking (%)	20
Diabetes mellitus (%)	40
Dyslipidemia (%)	85
Previous cardiovascular disease (%)	20
Clinic blood pressure	
SBP (mmHg)	151 ± 15
DBP (mmHg)	81 ± 8
Glucose-lipid metabolism	
Fasting plasma glucose (mg/dL)	130 ± 51
Glycated hemoglobin (%)	6.1 ± 0.8
Total cholesterol (mg/dL)	230 ± 33
LDL-cholesterol (mg/dL)	143 ± 27
HDL-cholesterol (mg/dL)	63 ± 21
Triglycerides (mg/dL)	195 ± 140
Renal function	
Serum creatinine (mg/dL)	1.04 ± 0.44
eGFR (mL/min/1.73 m^2)	60.4 ± 25.8
UACR (mg/g-Cr)	1034 ± 1480
Serum cystatin C (mg/L)	1.30 ± 0.45
Inflammatory and oxidative stress markers	
hs-CRP (mg/dL)	0.18 ± 0.24
Serum pentosidine (μg/mL)	0.036 ± 0.013
Medication	
Statin (%)	30
Calcium channel blockers (%)	100
RAS inhibitors (%)	85

Values are means ± SD. SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; UACR: urine albumin-to-creatinine ratio; CRP: C-reactive protein; RAS: renin-angiotensin system.

t-test. Univariate and multivariate linear regression analyses were performed to identify the factors affecting the changes in cSBP and UACR. The independent variables entered into the multivariate model were those that were significantly associated in the univariate analyses or were significantly different after a period of 16-week treatment. Analysis was performed using SPSS version 19.0 (IBM Corporation), and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Patient Characteristics. Table 1 shows the baseline characteristics of the total 20 hypertensive CKD patients enrolled from September, 2012, to November, 2013. The causes of CKD were hypertensive nephrosclerosis ($n = 11$), diabetic nephropathy ($n = 6$), chronic glomerulonephritis ($n = 2$), and obstructive nephropathy due to urinary tract stones ($n = 1$). None of the enrolled patients discontinued participation in the study. Mean age was 67.7 ± 12.4 years, and the number of males and females was 14 and 6, respectively. Body mass index was 26.8 ± 4.9 kg/m^2, suggesting that the participants correspond to obese hypertensive patients as a whole. With respect to BP control, clinic BP did not at all achieve the target BP level according to the JSH2009 guideline (clinic SBP/DBP 151 ± 15/81 ± 8 mm Hg) [10].

Table 2: Effects of single-pill amlodipine/atorvastatin-based therapy on clinic blood pressure profile.

	Baseline	Week 16
Clinic blood pressure		
SBP (mmHg)	151 ± 15	131 ± 11**
SBP-SD	6.1 ± 2.5	3.1 ± 2.2**
SBP-CV (%)	4.1 ± 1.6	2.4 ± 1.8**
DBP (mmHg)	81 ± 8	74 ± 10**
DBP-SD	4.6 ± 2.6	2.3 ± 1.7**
DBP-CV (%)	5.6 ± 3.1	3.1 ± 2.3*
Pulse rate (beat/min)	74 ± 7	69 ± 7**

Values are means ± SD. SBP: systolic blood pressure; DBP: diastolic blood pressure. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2. Effects of Combination Therapy with Amlodipine and Atorvastatin on BP Profile, Glucose and Lipid Metabolism, and Inflammatory and Oxidative Stress Markers. As a whole, the combination therapy with amlodipine and atorvastatin for 16 weeks significantly decreased clinic SBP and DBP (Table 2), and clinic pulse rate was also reduced by the combination therapy (Table 2). In addition, achievement of target BP control, which was defined as BP values less than 130/80 mmHg for CKD patients according to the JSH2009, was attained in an average of 45% after the combination therapy in spite of the presence of no achievement at baseline. Furthermore, the combination therapy significantly decreased the within-visit SBP and DBP variability (BP-SD and BP-CV) (Table 2).

With respect to the glucose and lipid metabolism, the combination therapy with amlodipine and atorvastatin significantly lowered total and LDL-cholesterol levels, without evident changes in parameters of glucose metabolism and inflammation (Table 3). In addition, achievement of the lipid goal, which was defined as LDL-cholesterol level less than 100 mg/dL, was attained in an average of 70% after the combination therapy in spite of the presence of no achievement at baseline. On the other hand, the serum pentosidine level was significantly increased by the combination therapy (Table 3). During a period of 16-week treatment, only two patients who could not achieve the BP goal by the maximum dose of amlodipine (10 mg/day) were additionally administered valsartan 80 mg/day and hydrochlorothiazide 12.5 mg/day, respectively. On the other hand, none of patients was additionally administered lipid-lowering drugs other than atorvastatin. The average doses of amlodipine and atorvastatin were 7.1 ± 2.6 mg/day and 7.0 ± 3.0 mg/day, respectively.

Table 3: Effects of single-pill amlodipine/atorvastatin-based therapy on glucose-lipid metabolism and inflammatory and oxidative stress markers.

	Baseline	Week 16
Glucose-lipid metabolism		
Fasting plasma glucose (mg/dL)	130 ± 51	127 ± 56
Glycated hemoglobin (%)	6.1 ± 0.8	6.2 ± 0.9
Total cholesterol (mg/dL)	230 ± 33	180 ± 37**
LDL-cholesterol (mg/dL)	143 ± 27	97 ± 30**
HDL-cholesterol (mg/dL)	63 ± 21	61 ± 19
Triglycerides (mg/dL)	195 ± 140	180 ± 134
Inflammatory and oxidative stress markers		
hs-CRP (mg/dL)	0.18 ± 0.24	0.09 ± 0.10
Serum pentosidine (μg/mL)	0.036 ± 0.013	0.046 ± 0.018**

Values are means ± SD. CRP: C-reactive protein. ** $P < 0.01$.

Table 4: Effects of single-pill amlodipine/atorvastatin-based therapy on renal and vascular functions.

	Baseline	Week 16
Renal function		
Serum creatinine (mg/dL)	1.04 ± 0.44	1.09 ± 0.50
eGFR (mL/min/1.73 m^2)	60.4 ± 25.7	58.8 ± 25.6
UACR (mg/g-Cr)	1034 ± 1480	733 ± 1218*
Serum cystatin C (mg/L)	1.30 ± 0.45	1.31 ± 0.49
Vascular function		
baPWV (cm/s)	1903 ± 353	1786 ± 382*
AI (%)	84 ± 15	81 ± 14
cSBP (mmHg)	148 ± 19	129 ± 23**

Values are means ± SD. eGFR: estimated glomerular filtration rate; UACR: urine albumin-to-creatinine ratio; baPWV: brachial-ankle pulse wave velocity; AI: augmentation index; cSBP: central systolic blood pressure.
* $P < 0.05$, ** $P < 0.01$.

3.3. Effects of Combination Therapy with Amlodipine and Atorvastatin on Renal and Vascular Function Parameters. Combination therapy with amlodipine and atorvastatin for 16 weeks significantly decreased UACR (Table 4; UACR, 1034 ± 1480 versus 733 ± 1218 mg/g-Cr, $P < 0.05$). In addition, the reduction of UACR by the combination therapy was not accompanied with decline in eGFR (Table 4; eGFR, 60.4 ± 25.7 versus 58.8 ± 25.6 mL/min/1.73 m^2). Concerning parameters of vascular function, the combination therapy with amlodipine and atorvastatin for 16 weeks significantly improved both baPWV and cSBP (Table 4; baPWV, 1903 ± 353 versus 1786 ± 382 cm/s, $P < 0.05$; cSBP, 148 ± 19 versus 129 ± 23 mmHg, $P < 0.01$).

3.4. Multivariate Regression Analysis for Assessment of Factors Contributing to Improvements in Central BP and Albuminuria. Multivariate regression analysis of the independent variables, which showed significant correlations in the univariate analyses or were significantly different after a period of 16-week treatment, indicated that there were significant associations between the changes in cSBP and those in SBP and total cholesterol and changes in UACR and those in serum creatinine and total cholesterol (Table 5).

Table 5: Multivariate linear regression analyses of factors associated with changes in cSBP and UACR.

Variables	β	P value
Change in cSBP (mmHg)		
Change in SBP (mmHg)	0.654	0.001
Change in total cholesterol (mg/dL)	0.439	0.020
(Model $R^2 = 0.679$)		
Change in UACR (mg/g-Cr)		
Change in SBP (mmHg)	−0.027	0.885
Change in serum creatinine (mg/dL)	−0.729	0.002
Change in total cholesterol (mg/dL)	0.418	0.034
(Model $R^2 = 0.643$)		

R^2: coefficient of determination. cSBP: central systolic blood pressure; UACR: urine albumin-to-creatinine ratio. These values are adjusted by age and sex.

4. Discussion

The main finding of this study was that the single pill-based administration for combinatorial antihypertensive and LDL cholesterol-lowering therapy with amlodipine and atorvastatin successfully decreased clinic BP so as to improve the achievement of target BP in hypertensive CKD patients already being treated before the start of the combination therapy. In addition, the combination therapy was able to significantly reduce UACR without decrease in eGFR and resulted in significant improvements in vascular function and lipid metabolism. These pleiotropic therapeutic effects by combination therapy with amlodipine and atorvastatin on biomarkers of renal and vascular damage deserve further discussion.

Recent clinical guidelines for hypertensive patients recommend combination therapy such as renin-angiotensin system inhibitors and CCB or diuretics, and, in this study, the combination therapy with amlodipine and atorvastatin was effective for efficient lowering of clinic BP and inhibition of within-visit BP variability in Japanese hypertensive CKD patients. With respect to BP-lowering efficacy of amlodipine, we previously showed that the amlodipine add-on group exerted greater reductions of clinic BP and home BP than the ARB add-on group [23].

Accumulated evidence indicates that renin-angiotensin system inhibitors such as ARB and ACE inhibitors are able to improve albuminuria better than CCB such as amlodipine through the reduction of intraglomerular pressure [24]. However, the combination therapy with amlodipine and atorvastatin exerted a significant reduction in albuminuria in the present study. Of note, in multivariate analysis, the decrease in total-cholesterol level was an independent contributing factor to the decrease in UACR. This result suggests that statin therapy may have a favorable effect on albuminuria and is confirmed with previous studies [25–28].

A previous study showed that the decreases in BP significantly contributed to the decreases in albuminuria by CCB in hypertensive and CKD patients [23, 29]. Although analysis of patient characteristics at baseline unexpectedly revealed that all participants were already treated with CCB in spite of only 30% of the participants being treated with statin, the combination therapy with amlodipine and atorvastatin for 16 weeks succeeded to efficiently suppress albuminuria, irrespective of preceding medication, without further decline in eGFR. This is likely to be an important advantage of the combination therapy with amlodipine and atorvastatin, since several recent epidemiological studies and intervention trials demonstrated that efficient reduction of albuminuria with preserved eGFR is important to inhibit the progression of CKD and to prevent the development of cardiovascular complication [30–32].

The combination therapy with amlodipine and atorvastatin exhibited a significant improvement in vascular functional parameters such as baPWV and cSBP in this study. A previous study showed that the add-on amlodipine therapy had benefits in terms of the vascular function and vascular structure of hypertensive patients already treated with renin-angiotensin inhibitors, which were independent of its depressor effects but with a concomitant decrease in ambulatory BP variability [33]. Also, these results are consistent with a previous study showing that coadministered amlodipine and atorvastatin produced early improvements in arterial wall compliance in hypertensive patients with dyslipidemia [34]. Since statins may exert their vascular beneficial effects by inhibiting small GTPase protein synthesis, such as Ras and Rho kinase [35, 36], the combination therapy of amlodipine and atorvastatin may have an additive beneficial effect on the vasculature so as to improve vascular atherosclerotic changes [8, 9].

Recently, parameters of BP variability obtained by clinic BP measurement are suggested to be associated with target organ damage including CKD and cardiovascular complication [37–39]. Variation in BP that is captured at sequential office visits is called visit-to-visit variability, and BP variation that is captured within each office visit is called within-visit BP variability. Accumulated evidence has indicated that greater degrees of clinic BP variability such as visit-to-visit BP variability and within-visit BP variability are associated with renal deterioration and cardiovascular complication [14, 37–40]. Almost all reported results showed that these parameters of BP variability, such as clinic BP variability and home-measured BP variability in addition to ambulatory BP variability, reflect organ damages and are potential predictors of cardiovascular events [37, 41, 42]. Furthermore, these analyses also displayed that CCB is the most effective drug class for reduction of BP variability [43].

A recent study showed that alteration of vascular function is an important factor in the correlation between clinic BP variability and cardiovascular disease [44], and clinic BP variability is recently suggested to correlate significantly with renal function and renal arteriosclerotic change as an emerging candidate of linking factor between vascular alteration and renal damage [38]. The results of the present study demonstrated that the combination therapy with amlodipine and atorvastatin significantly decreased clinic BP and its variability concomitant with improvements in parameters of vascular and renal function. In the present study, there was no significant relationship between the improvement in lipid metabolism and that in clinic BP and within-visit BP variability on univariate analysis (data not shown). Therefore, the association between statin therapy and BP or BP variability is still to be determined in the future study.

In the present study, pulse rate was significantly decreased by combination therapy of amlodipine and atorvastatin in spite of BP reduction. It has been reported that amlodipine tends to increase pulse rate via the reflex stimulation of sympathetic nervous system [45]. On the other hand, there are several previous studies suggesting that statins can reduce sympathetic activity, thereby resulting in pulse rate reduction [46, 47]. Thus, the decrease in pulse rate may be caused by concomitant statin therapy. However, since there is no direct data which support this possibility in the present study, further investigation is needed to elucidate this issue.

There are several limitations in the present study. Firstly, the present study is not randomized parallel-group control trial, and the numbers are small. Secondly, the patient's adherence was not examined in the present study. Since there are several previous studies suggesting that single-pill amlodipine/atorvastatin rather than 2-pill regimen may reduce cardiovascular events by improving the patient's adherence [48, 49], the improving effect of single-pill amlodipine/atorvastatin therapy on patient's adherence may have an influence on the results of this study. Further studies are needed to estimate the true long-term advantage of the amlodipine/atorvastatin combination in lowering blood pressure and improve vascular damage.

5. Conclusions

In summary, the results of the present study suggest that the combination therapy with amlodipine and atorvastatin may exert additional beneficial effects on renal and vascular damages as well as BP profile in addition to BP lowering in hypertension with CKD.

Disclosure

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Kengo Azushima and Kazushi Uneda equally contributed to this work.

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