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# Update on kidney transplantation in human immunodeficiency virus infected recipients

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## Abstract

Improved survival of human immunodeficiency virus (HIV) infected patients with chronic kidney disease following the introduction of antiretroviral therapy resulted in the need to revisit the topic of kidney transplantation in these patients. Large cohort studies have demonstrated favorable outcomes and proved that transplantation is a viable therapeutic option. However, HIV-infected recipients had higher rates of rejection. Immunosuppressive therapy did not negatively impact the course of HIV infection. Some of the immunosuppressive drugs used following transplantation exhibit antiretroviral effects. A close collaboration between infectious disease specialists and transplant professionals is mandatory in order to optimize transplantation outcomes in these patients. Transplantation from $HIV^+$ donors to $HIV^+$ recipients has been a subject of intense debate. The HIV Organ Policy Equity act provided a platform to research this area further and to develop guidelines. The first $HIV^+$ to $HIV^+$ kidney transplant in the United States and the first $HIV^+$ to $HIV^+$ liver transplant in the world were recently performed at the Johns Hopkins University Medical Center.

**Key words:** End-stage kidney disease; Human immunodeficiency virus; Antiretroviral therapy; Kidney transplantation



**Core tip:** Experience with kidney transplantation in human immunodeficiency virus (HIV) positive patients is evolving. With appropriate selection of candidates, the outcomes appear similar to that in HIV negative population. There are challenges with kidney transplantation in HIV positive patients including increased risk for acute rejection and drug-drug interactions. Optimal immunosuppressive regimen is unknown. This article discusses the recent advances in kidney transplantation among HIV positive patients.

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## INTRODUCTION

Human immunodeficiency virus (HIV) infection continues to be a healthcare problem worldwide. According to the Centers for Disease Control and Prevention, it is estimated that roughly 50000 people get infected with HIV each year in the United States. At the end of 2012, around 1.2 million people were living with HIV infection in the United States[1]. HIV infection used to be lethal in the past. The advent of highly active antiretroviral therapy resulted in a paradigm shift in that chronic illnesses now surpass opportunistic infections as causes of death in patients infected with HIV. Kidney disease continues to cause significant morbidity and mortality among the HIV infected population[2-4]. Currently, HIV-related nephropathies are considered as the third leading cause of end stage renal disease (ESRD) among African Americans[5,6].

There are several known etiologies for chronic kidney disease (CKD) in HIV infected patients. There is paucity of accurate epidemiological data due to the lack of renal biopsies performed in suspected cases and due to the inconsistent reporting of the disease. In the pre anti-retroviral therapy (ART) era, HIV associated nephropathy (HIVAN) used to be the most common cause of CKD in HIV infected patients affecting primarily African Americans. However more recently, hypertension, diabetes mellitus and cardiovascular disease evolved as significant causes of renal dysfunction in this patient population. HIV associated immune complex-mediated disease, IgA nephropathy, HIV-associated thrombotic microangiopathy and antiretroviral medication related toxicities are also important etiologies for CKD. Furthermore, most HIV sero-positive patients are co-infected with hepatitis C virus which can also cause CKD[7]. Progression to ESRD in CKD patients who are also HIV infected is more rapid than those without HIV infection[8]. Kidney diseases associated with HIV infection are summarized in Table 1.

According to Medicare claims data, the number of prevalent HIV positive ESRD patients has increased more than 14-fold from 1999 to 2010[9]. Outcomes of HIV infected dialysis patients have improved dramatically[8,9]. Actual number of HIV positive patients who received kidney transplants or who are on the waiting list is unknown. This is due to the fact that the Organ Procurement and Transplantation Network (OPTN) does not collect data on HIV infection among wait-listed candidates, and some states prohibit the reporting of HIV status.

HIV was once an absolute contraindication for kidney transplantation; however recent studies highlight the safety of kidney transplantation in HIV positive patients who are well-controlled on ART. Several challenges continue to exist in this area including choice of immunosuppression drugs, drug-drug interaction and heightened risk of infections. Furthermore, the possibility of considering HIV positive donors has been a topic of discussion in the recent years in order to allow an increase in the donor pool as discussed later.

## ACCESS TO TRANSPLANTATION IN HIV INFECTED PATIENTS

Historically, HIV infected patients were excluded from the consideration for kidney transplantation due to the concern for worsening infections and rejection. Hemodialysis and peritoneal dialysis were the only forms of treatment available for these patients[10]. In a survey of 148 United States transplant centers published in 1998, the majority of responding centers would not transplant kidney from deceased (88%) or living (91%) donors into HIV-infected patients. Most centers feared that transplantation in such patients would be harmful to the recipient, and some believed that it would be a waste of scarce donor organs[11]. However, recent studies have demonstrated that kidney transplantation in HIV positive patients with ESRD who are receiving ART is safe and effective. Outcomes were comparable to recipients without HIV infection. Furthermore, HIV positive individuals have higher waitlist mortality rates than their HIV negative counterparts. This along with an understanding of the role of immune activation in HIV disease pathogenesis and how immunosuppressant drugs exert antiviral effects contributed to a renewed interest in studying the outcomes of transplantation in these patients[8].

## OUTCOMES OF KIDNEY TRANSPLANTATION IN HIV INFECTED PATIENTS

Early experience with kidney transplantation in HIV positive patients before the rollout of ART was disappointing. This experience was based on case reports and small series of patients with short follow up[12,13]. Among 39 kidney transplants in HIV positive patients between 1980 and 1996, outcomes were suboptimal with 21 deaths after a mean follow up of 48 mo[14]. These included cases where HIV was transmitted during kidney transplant. Retrospective analysis of the United States Renal Data System database from 1987 to 1997 demonstrated inferior three and five-year graft and five-year patient survivals in HIV positive deceased donor kidney transplant recipients as compared to HIV negative patients[15].

Following the introduction of ART, several small studies showed encouraging patient and allograft survivals. The largest prospective trial of kidney transplantation in HIV-infected patients was conducted by Stock *et al*[16] and included 150 patients who were followed for up to five years at 19 United States transplant centers. This study showed one and three-year patient survival rates of 94.6% and 88.2% respectively. Corresponding graft survival rates were 90.4% and 73.7% respectively.

**Table 1 Causes of kidney disease in human immunodeficiency virus infected patients**

| Cause | Characteristics |
|---|---|
| HIVAN | Collapsing glomerulopathy in the setting of high grade HIV viremia<br>Affects almost exclusively African Americans<br>Manifests with high-grade proteinuria in the absence of hypertension<br>Treated with antiretroviral therapy |
| HIV-immune complex | Manifests with hematuria and sub-nephrotic range proteinuria<br>Variable presentation with AKI<br>Poorly understood |
| Diabetic nephropathy | Similar presentation to patients without HIV. Proteinuria followed by decreased GFR |
| Hypertension | Similar presentation to patients without HIV |
| Thrombotic microangiopathy | Typically presents with AKI, subnephrotic range proteinuria with hematuria along with features of microagiopathic hemolytic anemia |
| IgA nephropathy | Hematuria with variable degree of proteinuria and decreased GFR |
| Tenofovir toxicity | Variable degree of decreased GFR with features of proximal tubular injury |
| Immune-complex membrano-proliferative glomerulonephritis and cryoglobulinemia in the setting of HCV co-infection | Nephritic syndrome picture with positive cryoglobulin and hypocomplementemia |

HIV: Human immunodeficiency virus; HIVAN: HIV-associated nephropathy; AKI: Acute kidney injury; HCV: Hepatitis C virus.

These rates were generally between those reported in the Scientific Registry of Transplantation Recipients (SRTR) database for kidney transplant recipients ⩾ 65 years and all kidney transplant recipients during a similar time frame. However, there were higher rates of acute rejection at one year (31%) and three years (41%)[16]. In a study that included 40 HIV positive patients, Kumar *et al*[6] reported one and two year patient survival rates of 85% and 82% respectively. Corresponding graft survival rates were 75% and 71% with a 22% acute rejection rate. HIV viral load remained undetectable and CD4 T-cell counts were > 400 cells/mm$^3$. No opportunistic infections or progression to AIDS up to 2 years were observed in these patients[6]. Patient and graft survival rates were similar to HIV negative patients in the study by Roland *et al*[17] involving 18 HIV positive kidney transplant recipients with median follow up of 3.4 years. In a retrospective review of the UNOS database from 2004 to 2006, no differences in patient survival were observed between 100 HIV positive and 36492 HIV negative kidney transplant recipients (95.4% *vs* 96.2%, $P$ = 0.32). However, death-censored graft survival was significantly lower in the HIV positive patients (87.9% *vs* 94.6%, $P$ = 0.03). Donor age, cold ischemia time of at least 16 h and delayed graft function were associated with a greater than four-fold increase in allograft loss among the HIV positive patients[18]. A recent study reported 10 year outcomes of kidney transplantation in HIV positive patients from 2002 to 2012 using the SRTR database. When risk stratified by hepatitis C virus (HCV) infection status, monoinfected HIV positive recipients had similar five-year (75.0% *vs* 75.8%, $P$ = 0.58) and 10-year (55.9% *vs* 56.0%, $P$ = 0.49) graft survivals when compared to matched controls who were negative for both HIV and HCV. On the contrary, patients coinfected with HIV and HCV had inferior five-year (52.0% *vs* 64.0%, $P$ = 0.02) and 10-year (27.0% *vs* 36.2%, $P$ = 0.004) graft survival rates when compared to HIV negative but HCV positive matched controls. Coinfection with HCV, panel reactive antibodies > 80%, acute rejection episodes and cold ischemia time > 10 h were independent risk factors for graft loss. Patient survivals were higher in monoinfected HIV positive recipients at five-years (88.7%) and 10-years (63.5%). On the other hand, patient survivals were inferior among coinfected HIV positive recipients ($HV^+/HCV^+$) at 5-year (66.3%) and 10-year (29.3%)[19]. Mate kidney analyses using SRTR database from 2000 to 2013 showed similar long term outcomes of kidney transplantation in HIV positive patients relative to noninfected recipients. HIV and HCV coinfected patients had inferior outcomes in this analysis[20].

European transplant centers have similar experience to that in the United States. In a series of 27 HIV infected patients who received kidney transplant, two-year patient and graft survival rates were 98% and 96% respectively. Acute rejection rate was at 15% which is lower than what was reported in the United States. Most patients in this study received basiliximab induction followed by maintenance with tacrolimus, mycophenolate mofetil (MMF) and steroids[21]. A more recent study from the United Kingdom included 33 HIV infected patients, 50% of whom received living donor kidneys and underwent induction with interleukin-2 (IL-2) receptor antibody and were maintained on triple immunosuppression. Three year patient and allograft survival rates were 91.3% and 87.4% respectively. Acute rejection rate was 44% and 2 patients developed BK nephropathy[22].

## LISTING CRITERIA FOR HIV POSITIVE PATIENTS

Data regarding the evaluation of HIV infected patients for kidney transplant is limited. It is believed that, compared to HIV negative patients, only a smaller percentage of HIV infected patients evaluated for kidney transplantation are actually placed on the list. Barriers to listing for transplant were discussed in a retrospective study of 309 HIV infected patients evaluated for renal transplantation in one United States center between 2000 and 2009. Only 20% were listed for transplant compared with 73% in HIV negative patients evaluated during the same period ($P$ < 0.00001). The most common reason for not advancing the evaluation process was the lack of documentation of HIV control. CD4 T-cell count and viral load data were not

**Table 2 Inclusion criteria for kidney transplant listing in human immunodeficiency virus positive patients**

| |
|---|
| Meet standard criteria for placement on transplant waiting list for kidney transplantation plus the following |
| Well-controlled HIV disease with viral load < 50 copies/mL and CD4 count > 200 cells/mm$^3$ |
| Absence of opportunistic infections or neoplasms |
| Stable antiretroviral regimen |
| Psycho-social clearance with demonstration of no active history of drug and/or alcohol use. Patients on stable methadone maintenance program can be considered |

HIV: Human immunodeficiency virus.

available in 35% of patients and in 21%, CD4 T-cell count and viral load did not meet the eligibility criteria. Other factors associated with incomplete evaluation process were Black race and history of illicit drug use[23].

The European experience was slightly different, and data from the EuroSIDA cohort study included 88 HIV infected ESRD patients. Inappropriate levels of CD4 T cell count and viral load were reported in 30% of cases and two-thirds of patients were excluded because of cardiovascular diseases or diabetes[24]. Generally accepted criteria for listing HIV positive patients for kidney transplantation are shown in Table 2[25,26]. An exception is usually given to certain treatable and preventable infections such as tuberculosis, esophageal candidiasis, and Pneumocystis jiroveci pneumonia.

## SPECIAL CONSIDERATIONS AND CHALLENGES FOR KIDNEY TRANSPLANTATION IN HIV-INFECTED PATIENTS

### *Donor factors*

In the past, most kidney transplants done for HIV infected patients were from deceased donors. However a report of 48 living donor transplants showed improved outcomes and less rejection rates[16]. Therefore, it is possible to proceed with kidney transplantation from living donors; however, donors need to be informed with the challenges associated with transplanting HIV positive recipients.

### *Infections*

It appears that the degree of immunosuppression from drug therapy and HIV itself does not necessarily lead to increased risk of infectious complications following transplantation in appropriately selected HIV positive candidates. Studies did not show increased incidence of opportunistic infections in HIV infected patients who underwent kidney transplantation[17].

### *Rejection*

Most studies reported higher rates of acute rejection compared to HIV negative recipients. In a retrospective analysis of the SRTR database, 516 HIV infected kidney transplants performed between 2003 and 2011 were compared to uninfected counterparts within the same period. Rates of acute rejection within the first year were 15% compared to 8% in the control group[27]. Although this did not affect short-term graft survival in these studies, it merits further studying as it may impact long term graft function. The two variables in clinical studies that were frequently associated with increased risk for acute rejection were deceased donor organs and the use of cyclosporine. One hypothesis was that perhaps the use of ART with potential interaction with calcineurin inhibitors (CNIs) may have resulted in subtherapeutic blood levels of CNIs. It is also possible that intense immunosuppression was deliberately avoided in these patients to prevent infectious complications as noted in the multicenter study reported by Stock *et al*[16]. HIV contains host HLA molecules which can increase the risk for allosensitization. HIV infected recipients may also have increased memory cell phenotype. However, a report by Canaud *et al*[28] may provide a better explanation of the high rates of rejection. In this study, authors performed protocol renal transplant biopsies on 19 recipients with HIV infection who had undetectable plasma level of HIV-1 RNA. It was found that HIV-1 infected the allograft in 68% of these patients. In 62% of instances, infection was located in the podocytes while remaining 38% of the infection was located in tubular cells. Podocyte infection was associated with faster deterioration of allograft function and nephrotic range proteinuria. It was suggested that perhaps this infection may stimulate the immune system *via* recruitment of inflammatory cells and cause cross reactivity with alloantigen and therefore be partially responsible for acute rejection[28]. The authors also developed a non-invasive test for HIV infection of the allograft by performing quantitative PCR of HIV RNA and DNA in the urine. Results correlated well with biopsy findings.

### *Kidney infection with HIV*

HIV-associated nephropathy is a well-described aggressive form of focal segmental glomerulosclerosis where the HIV directly infects the kidney cells. Specialized immuno-cytochemistry studies demonstrate the presence of the HIV core protein (p24) and the envelope glycoprotein (gp120) implicating infection of renal cells by HIV[29]. Past studies using in situ hybridization and PCR have demonstrated that HIV-1 can directly infect renal epithelial cells which act as a reservoir for HIV[29]. In the transplanted kidney, reinfection with HIV can occur early on after transplant and in the absence of HIV viremia. The mechanism is not well understood, however it is hypothesized that the virus is translocated from the recipient T-cells to the donor kidney cells. Unlike native kidney HIVAN, transplanted kidney did not demonstrate similar pathological appearance. Podocyte infection and tubular reinfection were the two

salient features of HIV infection of the allograft[28].

## IMMUNOSUPPRESSANT DRUGS

The early studies of kidney transplantation in HIV positive patients used no induction immunosuppression and maintenance therapy with cyclosporine and MMF. More than half of the patients developed acute rejection requiring treatment with anti-thymocyte globulin[6]. As mentioned earlier, some immunosuppressive drugs including CNIs, MMF and rapamycin, have shown efficacy against HIV with reduced viral replication. There are no studies comparing tacrolimus *vs* cyclosporine in this setting. Retrospective analysis showed that cyclosporine was associated with a higher incidence of rejection. On the other hand, some centers prefer cyclosporine over tacrolimus due to the diabetogenic effect of tacrolimus which can be enhanced by protease inhibitors (PIs).

Immunosuppressive drugs may exert antiviral effects, either by reducing cellular targets for the virus, or *via* direct antiviral effects[30]. For instance, cyclosporine can interfere with HIV gag processing. MMF interacts with nucleoside reverse transcriptase inhibitors (NRTIs) like abacavir, didanosine and tenofovir thus potentiating their anti-viral effects[31-33]. It is also thought that sirolimus may be associated with downregulation of the CCR5 receptor which may decrease HIV infectivity[34]. Sirolimus is less nephrotoxic than CNIs and is an effective anti-proliferative agent that could be beneficial against Kaposi's sarcoma[35]. Glucocorticoids are inducers of CYP 450 system. They can also increase $CD4^+$ T cell population, suppress HIV viral load and inhibit cytokine CCL2. As steroids are tapered following kidney transplantation, CD4 count may decrease and CNI level may go up. This may result in enhance CNI toxicity and possibility of infections. Close monitoring is therefore recommended[36,37].

In terms of induction therapies, monoclonal anti-interleukin-2 receptor antibodies have been shown to enhance CD4 T-cell counts. No negative outcomes associated with their use have been reported. On the other hand, several issues were reported with the use of antilymphocyte polyclonal antibodies. Increased risk of infections and hospitalizations was reported with the use of Thymoglobulin in 11 HIV infected patients when it was used to treat rejection[38]. In the multicenter United States study that included 150 patients, administration of Thymoglobulin as induction therapy was associated with twice as many serious infections per follow up year compared to patients who did not receive this therapy[16].

Until further evidence becomes available, we recommend induction therapy using anti-IL-2 receptor monoclonal antibodies such as basiliximab. We recommend using tacrolimus plus MMF with or without steroids depending on immune risk. The use of Thymoglobulin is not contraindicated but it should be used with caution due to severe depletion of lymphocytes and the potential for severe thrombocytopenia.

## USE OF ART FOLLOWING TRANSPLANTATION

There are six classes of ART drugs currently available in the United States. These include nucleoside and non-nucleoside reverse transcriptase inhibitors (NNRTI), PIs, integrase strand-transfer inhibitors, CCR5 antagonists such as maraviroc and fusion inhibitors[39]. There is no consensus on the ideal ART regimen for kidney transplant recipients. It is generally recommended that patients continue the same ART regimen prescribed pre-transplant. Goal is the maintenance of HIV suppression while minimizing interaction with immunosuppressive drugs and their side effects. Multiple drug interactions exist between ART and immunosuppressive drugs. This is discussed in length below. Integrase strand transfer inhibitors such as raltegavir and dolutegravir have no interaction with immunosuppressive drugs at the CYP 450 level. It is recommended that they be used in combination with abacavir and lamivudine/emtricitabine. Renal dosing of medications is recommended as most kidney transplant recipients will have some degree of CKD. PIs and NNRTI are metabolized through liver and therefore do not require any dose adjustments. Raltegravir does not require renal dose adjustment either. ART that usually require renal dosing include nucleosides and nucleotides. Tenofovir can cause renal toxicity and should be avoided or used with caution in patients with kidney transplant. CCR5 chemokine receptor is used by R5 tropic virus for cell entry. Maraviroc blocks this receptor and can also impair lymphocyte chemotaxis with a theoretical reduction in organ transplant rejection[40]. Collaboration between infectious disease and transplant professionals with HIV viral load monitoring is essential in these cases[14].

## IMMUNOSUPPRESSION AND ART: DRUG-DRUG INTERACTIONS

Complex pharmacokinetic interactions between therapies used for immunosuppression and antiretroviral drugs can happen. MMF inhibits inosine monophosphate dehydrogenase which blocks purine synthesis. It is metabolized mainly by glucuronidation in the liver. Atazanavir, an inhibitor of UDP-glucuronosyl transferase may lead to increased mycophenolic acid (MPA) levels[14]. Ritonavir on the other hand, may reduce MPA levels by inducing glucuronidation. Drugs that affect cytochrome P-450 may also influence the levels of CNIs and sirolimus. For example, PIs inhibit CYP 450 and p-glycoprotein efflux system resulting in increased serum levels of CNIs. Patients on PIs may require only small doses of CNI given less frequently. Special attention should be given when stopping PIs in these patients as this may result in acute rejection[41-43]. On the other hand drugs in NNRTI group can reduce CNI serum levels due CYP 450 induction. Stopping NNRTIs may result in CNI toxicity[44]. Maraviroc, is a P-450 3A4 substrate, but does not inhibit or induce the enzyme and hence, it is not expected to interact with CNIs. Intergrase

**Table 3 Key points**

| |
|---|
| Kidney transplantation in patients with HIV infection is a viable therapeutic option |
| Ideal immunosuppressive regimen remains uncertain |
| Higher rates of rejection are reported in clinical trials |
| Immunosuppressive therapy does not seem to negatively impact the course of HIV infection |
| Some immunosuppressive drugs may exert antiretroviral actions |
| Special attention should be paid to the potential interaction between ART and immunosuppressive drugs |
| A close collaboration between infectious disease specialists and transplant professionals is mandatory in order to optimize transplantation outcomes in these patients |
| Transplantation from HIV+ donors to HIV+ is currently being researched |

HIV: Human immunodeficiency virus.

strand transfer inhibitors such as raltegravir, has excellent anti-retroviral effects without affecting CYP system and hence no significant interaction with CNIs. This was studied by Tricot *et al*[45] in 5 patients who did not suffer any acute rejection. However lower barrier to resistance in this group of drugs may increase chances for virologic failure.

In addition to ART and immunosuppressive drug-drug interactions, several antibiotics and antifungal drugs used for treatment and prevention of infections in HIV patients can inhibit cytochrome P450 system and hence affect the CNI levels.

The complexity of drug-drug interactions highlights the importance of team approach that includes transplant nephrology, infectious disease and specialized pharmacy.

## PATIENTS COINFECTED WITH HIV AND HCV

As mentioned, outcomes were inferior with kidney transplantation in patients coinfected with HIV and HCV when compared to HIV monoinfected transplant recipients[19,20]. Factors contributing to this may include HCV infection related increased risk for the development of post-transplant diabetes mellitus, liver damage, cardiovascular disease and infections. Coinfected patients may represent a social and biological high risk group. For instance, these patients generally are younger with lower income, have longer HIV disease duration and dialysis vintage prior to transplantation with greater likelihood of drug addiction history[20]. This raises the question whether HCV coinfection should be a relative contraindication for kidney transplantation in HIV positive patients. However over the last couple of years, there have been significant advances in the treatment of HCV infection with the introduction of directly acting antiviral agents (DAA) into the clinical arena[46]. These agents can achieve a sustained virologic response in the range of 90%-95% with minimal side effects. Moreover, unlike interferon based therapy, DAA are safe to use after organ transplantation. These therapeutic advances are likely to improve long-term outcomes in HCV infected organ transplant recipients.

## HIV TO HIV TRANSPLANTATION

A study from South Africa by Muller *et al*[47,48] reported the outcomes in 27 HIV positive patients who received deceased donor kidneys from HIV positive donors. All donors had normal kidney function and all kidneys were biopsied. At one, three and five years after transplant, patient survival rates were 84%, 84% and 74% respectively with corresponding death-censored graft survival rates of 93%, 84% and 84%. HIV viral loads remained suppressed without evidence for opportunistic infections during the follow-up in all patients. Three patients developed HIVAN in the transplanted kidneys on protocol biopsies despite the lack of HIV viremia[47,48]. Whether the South African experience can be applied to the United States is not fully clear. In addition to the ethical dilemmas, concerns include possibility of superinfection with more virulent strains and development of drug resistance[49,50]. Viral tropism is another concern with theoretical risk for super infection with a more aggressive strain such as X4 tropic virus compared to R5 tropic virus. Tropism studies are available but may take up to a week to complete making it less useful for decision making during the narrow time window available to make transplant decisions[40]. Quality of donor organs and the risk for recurrence of HIVAN are also potential issues in HIV to HIV transplantation.

On November 21, 2013, President Obama signed the HIV Organ Policy Equity (HOPE) Act into law. This law reversed the federal ban on considering HIV positive donors and authorized clinical research in the area of transplantation from HIV positive organ donors[51]. As a result, a work group from the OPTN was charged with the development of policies that permit safe recovery of such organs. OPTN granted permission to Johns Hopkins University Hospital, Baltimore, MD to perform organ transplantation between HIV positive donors and recipients as of February 9, 2016. The transplant team at this center now has performed the first $HIV^+$ to $HIV^+$ kidney transplant in the United States and the first $HIV^+$ to $HIV^+$ liver transplant in the world. Experts estimate that using HIV infected donors will make available an additional 500 solid organ donors a year[52,53]. Moreover, this may reduce the discard of organs due to false positive results from nucleic acid testing currently being used which has false positive rates between 0.1% and 0.85%.

## CONCLUSION

Key point regarding kidney transplantation in HIV infected patients are summarized in Table 3. Evidence thus far supports the viability of kidney transplantation in appropriately selected HIV positive patients with acceptable outcomes. Ideal immunosuppressive regimen is not yet defined in this population. Special attention should be paid

to potential drug interactions between some of the ART medications and immunosuppressive drugs. Studies have shown increased incidence of acute rejection episodes and achieving therapeutic CNI levels can be challenging especially if the patient is on ART regimens which include PIs and NNRTIs. ART regimens containing integrase strand transfer inhibitors such as raltegravir may be preferred due to minimal drug interactions. Patients coinfected with HIV and HCV have inferior outcomes with kidney transplantation. However, outcomes are likely to improve in these patients in the coming years corresponding with the availability and use of DAA to treat HCV infection. The option for HIV positive donor to HIV positive recipient organ transplantation is actively researched in the United States and could further expand donor pool for HIV infected patients.

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REVIEW

# Reclassification of membranoproliferative glomerulonephritis: Identification of a new GN: C3GN

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## Abstract

This review revises the reclassification of the membranoproliferative glomerulonephritis (MPGN) after the consensus conference that by 2015 reclassified all the glomerulonephritis basing on etiology and pathogenesis, instead of the histomorphological aspects. After reclassification, two types of MPGN are to date recognized: The immunocomplexes mediated MPGN and the complement mediated MPGN. The latter type is more extensively described in the review either because several of these entities are completely new or because the improved knowledge of the complement cascade allowed for new diagnostic and therapeutic approaches. Overall the complement mediated MPGN are related to acquired or genetic cause. The presence of circulating auto antibodies is the principal acquired cause. Genetic wide association studies and family studies allowed to recognize genetic mutations of different types as causes of the complement dysregulation. The complement cascade is a complex phenomenon and activating factors and regulating factors should be distinguished. Genetic mutations causing abnormalities either in activating or in regulating factors have been described. The diagnosis of the complement mediated MPGN requires a complete study of all these different complement factors. As a consequence, new therapeutic approaches are becoming available. Indeed, in addition to a nonspecific treatment and to the immunosuppression that has the aim to block the auto antibodies production, the specific inhibition of complement activation is relatively new and may act either blocking the C5 convertase or the C3 convertase. The drugs acting on C3 convertase are still in different phases of clinical development and might represent drugs for the future. Overall the authors consider that one of the principal problems in finding new types of drugs are both the rarity of the disease and the consequent poor interest in the marketing and the lack of large international cooperative studies.

**Key words:** Glomerulonephritis reclassification; Dense deposit disease; Membranoproliferative glomerulonephritis; C3 glomerulopathies; Targeting complement pathways; Complement dysregulation

**Core tip:** The complement pathway dysregulation has been recognized as the main cause of some membrano-proliferative glomerulonephritis (MPGNs). This fact is at the basis of the new classification of the disease and of the findings of new entities as the complement factor H related protein nephropathy. Genetic studies as well as improvement in proteomics allowed recognizing the complement dysregulation as the cause of some renal diseases as the MPGN and the atypical hemolytic uremic syndrome that may be considered as strictly related diseases. The anti-complement drugs represent a new approach in the treatment of these diseases and their use in larger evidence based randomized trials is required.

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## INTRODUCTION

By 2015, nephrologists and renal pathologists held a consensus meeting to formulate a new etiology/pathogenesis-based system to classify glomerulonephritis (GN)[1]. According to the consensus report, GNs have been classified into five etiology/pathogenesis-based categories (Table 1).

According to the new classification, membrano-proliferative GNs (MPGN) have been reclassified and divided into different chapters on the basis of pathophysiology. In addition, new entities have been found. This review will discuss the new classification of MPGNs and will principally describe the complement-dysregulation dependent C3 glomerulopathies (C3G).

## MPGN

Until recently, the MPGNs have been distinguished according the histological and ultra structural findings and were classified as MPGN type Ⅰ, type Ⅱ and type Ⅲ. The glomerular lesions include mesangial hypercellularity, endocapillary proliferation and duplication of glomerular basement membrane (GBM) lesions[2]. Sub-endothelial and mesangial deposits are predominant in MPGN type Ⅰ[3]. Highly osmiophylic electron-dense intramembranous deposits characterize type Ⅱ GN[4], which is also known as dense deposits disease (DDD). In type Ⅲ MPGN deposits may be found in the sub-endothelial and sub-epithelial spaces[5].

With the discovery of the complement role in generating glomerular diseases[6], a new classification of MPGN was developed, based on pathophysiology and considering whether immunoglobulins accompany the complement using immunofluorescence on biopsy specimens[7,8] (Figure 1).

This new classification resulted in three principal consequences: (1) to identify new entities, which until now were unknown or misdiagnosed; (2) to highlight new diagnostic approaches. Indeed in the case of Ig-mediated MPGN, a work-up for infections, autoimmune diseases and monoclonal gammopathies should be adopted. In the case of complement-mediated GN, a complete study of the complement alternative pathway (AP) should be performed; and (3) to differentiate the therapeutic approach according to the type of MPGN. In summary, the three different forms of MPGN are now recognized as follows: (1) Immunocomplexes-associated MPGN with complement over activation (old MPGN type Ⅰ); (2) MPGN with intramembranous dense deposits (old MPGN type Ⅱ); and (3) C3GN, a new entity complement-mediated GN. DDD and C3GN are both related to complement dysregulation and are "*de facto*" included in the same chapter.

## IMMUNOCOMPLEXES ASSOCIATED MPGN

Immune-complexes mediated MPGN is caused by the deposition of immunocomplexes in the glomeruli. The immunocomplexes activate the classical pathway (CP) of complement and cause the deposition of complement factors or of the membrane attack complex (MAC) in the mesangium and capillary loops[9].

The MPGN is an uncommon cause of nephropathy (approximately 5 per million persons per year) and is more often secondary to infections, autoimmune disease and monoclonal gammopathy[10].

### *Pathophysiology*

**MPGN and infections:** Hepatitis C and B, which are often accompanied by circulating cryoglobulins, are a frequent cause of MPGN[11-14]. In addition, chronic bacterial infections, fungal and parasitic infections may also cause MPGN[15-17].

Immunocomplexes depositions are the first step. Consequently, CP is activated and in addition to the direct damage cause by MAC, C3a and C5a are generated that favor leukocyte accumulation, cytokine release and a further glomerular damage.

**MPGN and autoimmune diseases:** Mixed cryoglobulinemia is frequently associated with hepatitis C infection, systemic lupus erythematosus, sclerodermia, Sjögren syndrome and rheumatoid arthritis. These are the autoimmune diseases that more frequently cause MPGN due to the persistence of circulating immunocomplexes[18-21]. Under these conditions, circulating immunocomplexes may also activate the complement CP with the abovementioned subsequent events described for MPGN due to infections.

**MPGN and monoclonal gammopathy:** The renal deposition of monoclonal immunoglobulins (MIg) may determine a wide spectrum of renal lesions as recently

**Table 1 Classification of glomerulonephritis**

| Pathogenetic type | Specific disease entity | Pattern of injury: Focal or diffuse | Score or class |
|---|---|---|---|
| Immune-complex GN | IgA nephropathy, IgA vasculitis, lupus nephritis, infection-related GN, fibrillary GN with polyclonal Ig deposits | Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple | Oxford/MEST scores for IgA nephropathy ISN/RPS class for lupus nephritis |
| Pauci-immune GN | MPO-ANCA GN, proteinase 3-ANCA GN, ANCA-negative GN | Necrotizing, crescentic, sclerosing, or multiple | Focal, crescentic, mixed, or sclerosing class (Berden/ EUVAS class) |
| Anti-GBM GN | Anti-GBM GN | Necrotizing, crescentic, sclerosing, or mixed | |
| Monoclonal Ig GN | Monoclonal Ig deposition disease, proliferative GN with monoclonal Ig deposits, immunotactoid glomerulopathy, fibrillary GN with monoclonal Ig deposits | Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple | |
| C3 glomerulopathy | C3 GN, dense deposit disease | Mesangial, endocapillary, exudative, membranoproliferative, necrotizing, crescentic, sclerosing or multiple | |

GN: Glomerulonephritis; MEST: Mesangial hypercellularity, endocapillary hypercellularity, segmental sclerosis, interstitial fibrosis/tubular atrophy; ISN/RPS: International Society of Nephrology/Renal Pathology Society; MPO: Myeloperoxidase antibodies; ANCA: Antineutrophil cytoplasmic antibodies; EUVAS: European vasculitis study group; GBM: Glomerular basement membrane.

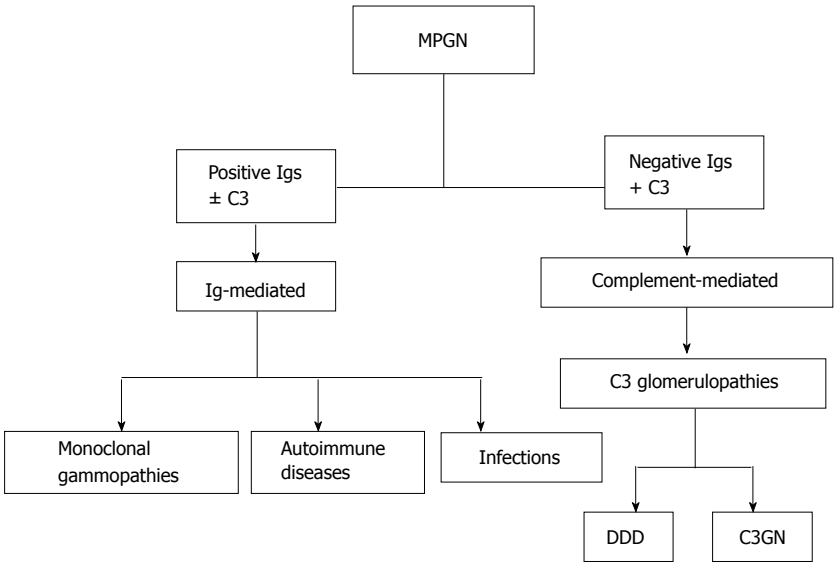


**Figure 1 Proposed classification for membranoproliferative glomerulonephritis based on the presence or absence of Igs and the presence of C3 by immunofluorescence.** MPGN: Membranoproliferative glomerulonephritis; Igs: Immunoglobulins; DDD: Dense deposit disease.

described[22]. Monoclonal gammopathy as well as light chain and heavy chain diseases may result in MPGN[23]. These lesions may hide a variety of severe hematological diseases ranging from low-grade B cell lymphoma, chronic lymphocyte leukemia to multiple myeloma[9]. In a recent monocenter study of MIg-associated MPGN after excluding infections and autoimmune disease, 26 out of 28 patients were serum electrophoresis-positive and 27 out of 28 patients were urine electrophoresis-positive[24]. In this monocentric study, out of 126 patients affected by MPGN, 41% were urine- or serum-positive for monoclonal gammopathy.

Monoclonal gammopathies are associated with complement activation; indeed, the abnormal immunoglobulin might activate the AP[10].

Recently, cases of C3 glomerulopathies, including C3GN and DDD (see below) associated with MIgs have been described[25]. In these patients the monoclonal immunoglobulin causes a complement dysregulation by interfering with the function of complement-regulating proteins, such as factor H or acting as an autoantibody against factor H or factor B[26-28].

### *Clinical and therapy*

Immunocomplex-mediated MPGNs principally affect children and young adults. Its clinical presentation may range from nephrotic syndrome and acute nephritic syndrome, to asymptomatic proteinuria and hematuria. Renal dysfunction frequently occurs, and 40% of the patients progress to end stage renal disease (ESRD) in approximately 10 years.

The efficacy of the different therapeutic approach is difficult to evaluate due to the small number of patients and because several trials include the three different types of MPGN[29,30]. The therapy most widely used is based on anti-cell proliferation agents[31]. Over-

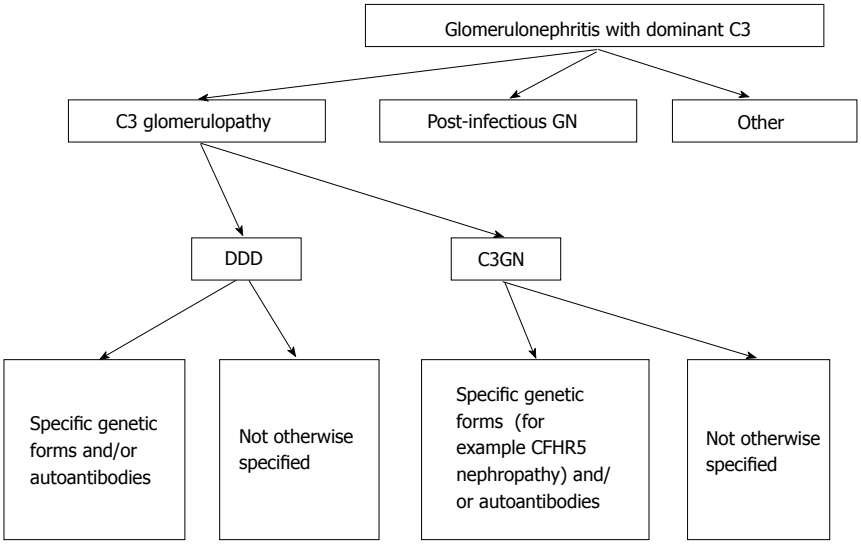


**Figure 2 Approach to the classification of glomerulonephritis with dominant C3.** DDD: Dense deposit disease; CFHR5: Complement factor H related protein.

activation of complement is often present, but whether anticomplement drugs might be useful in this context remains to be elucidated.

According to different studies, renal transplantation is a viable option in patients with ESRD, even if the disease recurs after transplantation with a frequency ranging from 27% to 65%[32-34]. In a recent study, after the exclusion of patients with DDD, a recurrence rate of 41% has been reported[34]. Such a high recurrence rate has been confirmed by a study published in 2016, which evaluated the recurrence rate using the new classification[35]. In another study the recurrence of Ig-mediated MPGN was lower (23.5%) and after a follow-up of 15 years, the graft survival rate of MPGN patients was similar to those of controls affected by different diseases[36].

## COMPLEMENT MEDIATED MPGN

MPGN patients that have on renal biopsy clear glomerular C3 staining with few or no immunoglobulin deposition are referred to as complement-mediated MPGN and are defined as C3G. C3Gs are less common than immune-complex-mediated MPGNs and are further divided into two groups according to the presence or absence of highly electron-dense deposits into the GBM.

The disease with intramembranous deposits corresponds to the DDD (previously called MPGN type Ⅱ). The disease without dense deposits, with C3 prevalence and no Igs on the glomeruli and with MPGN aspect on normal histology, is referred to a recently recognized entity: the C3GN. The distinction between the two diseases often requires the use of electron microscopy. C3GN was initially described by Servais *et al*[37] who described a series of 19 patients and proposed the term C3GN to highlight a disease that is characterized by C3 prevalence on the glomeruli, without intramembranous deposits. In addition, Servais *et al*[37] observed that this new entity often shares common genetic risk factors with atypical hemolytic uremic syndrome (aHUS).

Overall the term C3G was introduced to define all MPGNs that are characterized by the prevalence of C3 in the glomeruli[38], including DDD.

The term C3G has also been introduced because C3 isolated accumulation was recognized to include several heterogeneous entities and due to our improvement in the understanding of complement-mediated kidney injuries. Consequently, several complement factor abnormalities resulting in glomerular lesions have been identified. In 2013, a first consensus meeting on C3G was held to better clarify the pathogenic aspects and terminology[39]. The consensus conference resulted in an improved classification (Figure 2) that also documented that need of future work.

C3G are all caused by dysregulation of the complement AP and of the terminal complement complex (TCC)[40] (Figure 3).

### *Clinical features*

DDD has an estimated prevalence of 2 to 3 per million populations[41] and prevails in childhood and in young adults[42]. C3GN prevalence is difficult to be evaluated, as this disease is new and as time progresses, more patients are identified with family studies and with an improvement in the Genetic wide association studies.

Overall, patients affected by DDD are younger with respect to patients affected by C3GN[43]. Both diseases affect males and females with the same frequency[44-46]. Renal manifestations are similar in DDD and C3GN[43] and include hypertension, hematuria and proteinuria more often in the nephrotic range. Non renal manifestations of DDD include ocular lipoproteinaceous deposition and acquired lipodystrophy[47,48].

MIg in the serum may also be associated with both DDD and C3GN[25,49-51]. These patients often have a poor renal prognosis.

Progression to ESRD is common in both DDD and C3GN. Renal transplantation is feasible but with a high rate of disease recurrence[52].

Complement factor H related protein (CFHR5)

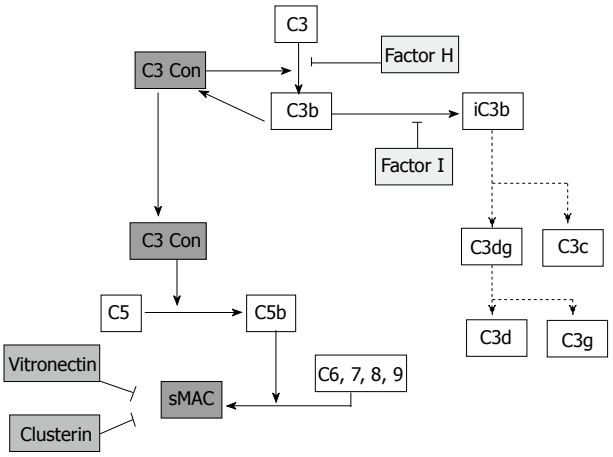


**Figure 3 Pathway of complement and complement regulator factors.** C3 Con: C3 covertase; sMAC: Serum membrane attack complex; C3dg, C3c, C3d, C3g: Complement degradation products.

nephropathy is a subtype that is well identified in C3GN caused by the presence of an abnormal CFHR5 protein. The disease is inherited and was first identified in Cypriot families[53]. The disease may often occur with clinical manifestations that are similar to IgA nephropathy with microscopic hematuria or macroscopic hematuria after an acute upper respiratory tract disease[54]. Progression to ESRD is common. Interestingly, ten patients affected by CFHR5 nephropathy received a successful renal transplantation[54].

### *Pathophysiology*

Dyregulation of the complement AP may occur principally due to acquired or genetic abnormalities[9] (Figure 4).

The auto-antibodies are the most frequently acquired abnormality. Auto-antibodies may be directed against the complement-regulating factors, such as factor H, factor I, factor B as well as against C3 convertase itself[55,56].

The first described autoantibody was the C3 nephritic factor (C3 NeF), which binds and stabilizes C3 convertase[57]. A second type of C3 NeF properdin dependent has also been described[58]. C3 NeFs are principally present in patients affected by DDD but are less frequently found in C3GN and absent in CFHR5 nephropathy[43].

In DDD, auto-antibodies that bind factor B and target C3B have been described in patients affected by MPGN type Ⅱ[56,59]. Anti CFH auto-antibodies have also been found in patients affected by DDD and C3GN[60,61].

Anti CFH auto antibodies are also frequently present in aHUS. A recent study[62] highlights that anti-factor H antibodies are equally present in C3G and aHUS, but that the auto-antibody structure is different in the two diseases. Indeed, in C3G, the auto-antibody principally binds to the amino terminal domains, while in aHUS, it binds to the carboxyterminal domain[10]. As previously mentioned, the two diseases are strictly related, but several differences are present.

The discovery of familial cases of C3G highlights that in several cases, a familial genetic basis of the disease occurs.

In 2010, Martínez-Barricarte *et al*[63] described a family in which some members were affected by a mutant form of C3 resistant to cleavage by C3 convertase. Consequently, this caused an AP dysregulation restricted to the fluid phase and these patients continuously produced and consumed C3 produced by the normal *C3* allele. These patients were affected by the classic DDD. Complement factor H-related (*CFHR)* genes are often involved. There are five CFH-related proteins (CFHR1-5 and genetic abnormalities of these proteins have been recognized and may cause disease. Recently, Chen *et al*[64] described two patients from the same family affected by DDD and with an abnormal deletion in the complement factor H-related (*CFHR*) gene cluster. This resulted in a hybrid CFHR protein that inhibited the complement decay-factor H-mediated.

Another genetic cause of C3G has been reported by Gale[53]. Gale *et al*[53] described two families of Cypriot origin whose members were affected by a mutation in CFHR protein 5. These patients were affected by a C3G that was defined as CFHR5 nephropathy. Indeed, genome-wide linked analysis (GWLA) allowed localization of a genetic abnormality in chromosome 1q31-32. In these patients, a larger CFHR5 protein is generated that is less effective in associating with surface-bound C3b. The resulting disease was known as CFHR5 nephropathy.

Recently, Malik *et al*[65] described an autosomal dominant complement-mediated C3G associated with abnormal copies in the *CFHR3* and *CFHR1* loci.

Finally, Habbig *et al*[66] described two siblings affected by renal disease. Both children had a homozygous deletion of 224 lysine of CFH. This deletion led to a defective complement control[67]. The renal disease was compatible with C3G. The authors proposed the name of C3 deposition glomerulopathy (C3DG) due to the absence of DDD.

Overall, these families highlight the genetic origin of several C3Gs related to a dysregulation of the AP and TCC.

Summarizing, the disease mechanisms in C3G caused by genetic defects identified in family studies may be classified into three categories: (1) homozygous deficiency dysfunction of CFH resulting in excessive C3 activation; (2) hyperfunctional C3 producing excessive C3 activation despite normal CFH activity; and (3) abnormal CFHR protein that enhances CFH dysregulation and consequent excessive C3 activation.

### *Diagnosis*

The diagnosis of C3G and differential diagnosis between DDD and C3GN should include a comprehensive pathological analysis and a complete work-up on the genetic and biochemical aspects of complement pathways, with particular regard to the AP.

Using light microscopy, in the case of C3 prevailing without Ig on glomeruli, only a suspicious diagnosis of

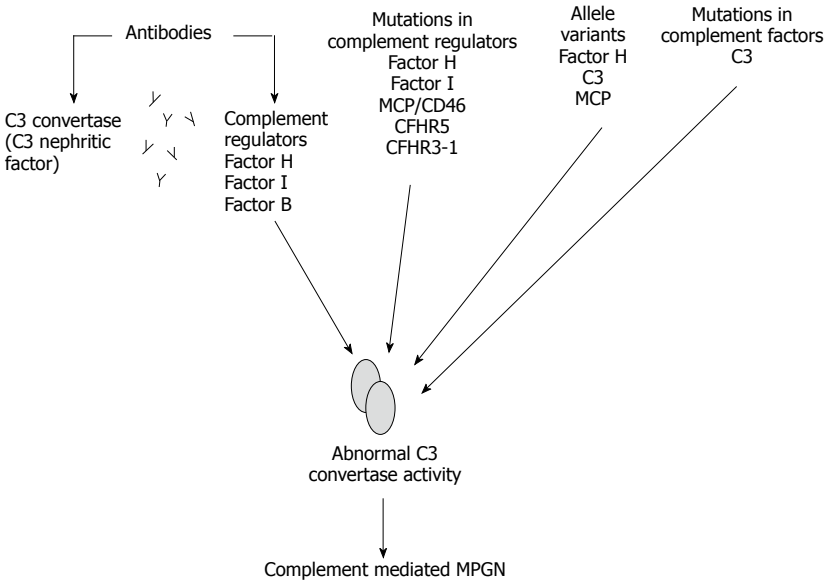


**Figure 4 Acquired and genetic abnormalities associated with complement-mediated membranoproliferative glomerulonephritis.** MCP: Membrane cofactor protein; CHFR: Complement factor H related proteins; MPGN: Membranoproliferative glomerulonephritis.

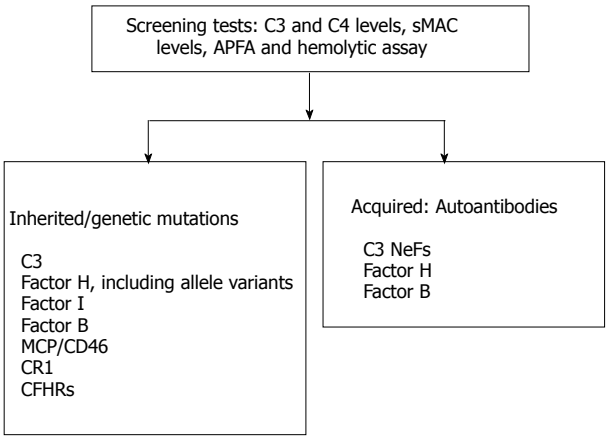


**Figure 5 Proposed work-up of complement mediated membranoproliferative glomerulonephritis.** APFA: Alternative pathway functional assay; CFHR: Complement factor H related proteins; CR1: Complement receptor 1; MCP: Membrane cofactor protein; sMAC: Serum membrane attack complex; MPGN: Membranoproliferative glomerulonephritis.

C3G may be formulated. The definitive diagnosis might only rely on ultra-structural basis.

Overall DDD, is characterized by dense osmiophilic band-like deposits within the GBM. C3GN may be characterized by sub endothelial and mesangial deposits, though intramembranous and sub epithelial deposits may also be present[68]. Several patients may present an overlap in the ultra-structural findings and are difficult to be classified. Proteomic studies may be useful for their identification[50,69].

The evaluation of the complement AP is essential for an improved diagnosis. The evaluation may be performed in several ways: (1) evaluating the total hemolytic complement assay[70]; (2) evaluating the complement alternative pathway assay[71]; and (3) evaluating the complement factor H functional assay[72].

In addition, the C3, C4 and serum MAC (sMAC) levels should be determined. In the case of positivity of these tests, genetic and enzyme-linked immunosorbent assays for complement abnormalities should be performed[8] (Figure 5).

Mutations in the *CFH*, *CFI* and *CD46* genes have been reported in some patients affected by DDD[39,43]. Changes in factor *B* and *C3* genes may also be present[56,63]. In CFHR5 nephropathy, an internal duplication in the *CFHR5* gene is present[53]. Other rearrangements of the *CFHR2-CFHR5* hybrid gene and other abnormalities in *CFHR1* and *CFHR5* have been reported[73-75].

An interpretation of identified variants may be difficult to be understood for several reasons[76]. The pathogenic variants accounts for only 25% of patients affected by DDD and C3GN[43,46]. In addition, mutations in other genes, such as thrombomodulin (*THBD*), diacylglycerol kinase-epsilon (*DGKE*), and the *CFHR* gene family have been recently found to be implicated to contribute to these diseases[77,78].

Moreover, further studies did not confirm a pathogenic role for several missense variants that were originally thought to be at the basis of the disease. Consequently, several amino acid changes in the gene structure are not "*de facto*" related to the disease[79].

Finally, most variants have a low penetrance and combined variants have been reported in 3% to 12% of patients[80].

The non-genetic causes of C3G are principally auto-antibodies: (1) C3 NeF: It binds directly to C3 convertase prolonging its survival. C3NeFs are found in 80% of patients affected by DDD and in 50% of patients affected by C3GN[43,59]. A C3NeF can stabilize C5 convertase in addition to C3 convertase, which has been previously described[81]. The detection of C3 NeF may be performed in several ways[82]. Further studies are needed to better correlate the presence of C3 NeF with the cause of the diseases and with treatment efficacy; (2) C4 NeF: The role of C4 NeF is still unclear, even if this auto-antibody has been found in some patients affected by MPGN[83]; (3)Anti-factor H auto-antibodies: Have been described

**Table 2 Complement testing in patients with C3 glomerulopathy**

| Test | Interpretation | Limitations |
|---|---|---|
| C3 and C4 levels | C3 frequently depressed and support diagnosis;<br>Normal C4 suggests an alternative pathway process | Non-specific |
| Soluble C5b-9 | May be indicator of active disease;<br>May identify patients who will benefit from C5 blockade | Test not widely available |
| C3 nephritic factor | Associated with C3 glomerulopathy;<br>May identify patients who will benefit from B cell targeted therapies | Levels do not correlate with disease activity; also seen in MPGN type I |
| Factor H protein levels | May identify underlying mechanism of alternative pathway activity;<br>May identify patients who will benefit from plasma infusion/exchange | |
| Autoantibodies to factor H and factor B | May identify underlying mechanism of alternative pathway activity;<br>May identify patients who will benefit from B cell targeted therapies | Test not widely available |
| Genetic mutation screening<br>Factor H<br>CFHR1, 2, and 5<br>Factor I<br>C3<br>Factor B | May identify underlying mechanism of alternative pathway activity | Not widely available;<br>Clinical implications unknown |

MPGN: Membranoproliferative glomerulonephritis; CFHR: Complement factor H related proteins.

**Table 3 Possible treatment of C3 glomerulopathies**

| |
|---|
| Nonspecific treatment |
| Replace deficient gene products |
| Plasma infusion |
| Liver Transplantation |
| Eliminate autoantibodies and/or mutant proteins |
| Plasma exchange |
| Immunosuppression |
| Treatment of plasma cell dyscrasia |
| Inhibition of complement activation |
| Eculizumab (anti C5) |
| Inhibition of the C3 Convertase |
| Renal transplantation |
| New trials ongoing |

in patients affected by DDD[41] and C3GN[46]. They may be detected using an enzyme-linked immunosorbent assay. If an anti-factor H is found, then monoclonal gammopathy should be excluded principally in older people[28,50]; and (4) Anti-factor B auto-antibodies are not frequently found and their research by enzyme-like immunosorbent assay is not easy and is often not available[56]. Overall, the suggested complement investigations in C3G are indicated in Table 2 as suggested by the previously cited consensus report[39].

### *Treatment*

Several treatments for C3Gs may be attempted. According to evidence-based medicine, to date, most of the treatments have not yet been proven to be effective in C3G (Table 3).

**Non-specific or supportive measures:** By extrapolating from the treatment of other chronic renal diseases, blood pressure control, reduction of proteinuria and the lowering serum lipid levels should have a beneficial effect in patients affected by C3G, and principally in those affected by a low disease progression[46].

In the previously mentioned French study[43], the renin-angiotensin-aldosterone system (RAAS) blockade was associated with prolonged renal survival, but these findings have not been confirmed by a United States study[49]. In the latter study, the RAAS blockade had beneficial effects only when associated with steroids. In another study, Maisch *et al*[84] documented the efficacy of a lipid-lowering strategy by statins.

**Replacement of deficient gene products:** Due to the unavailability of purified complement regulating factors, often a functioning factor may be administered by plasma infusion. The limitation is the need of lifelong substitution therapy.

Plasma infusion is not beneficial in patients affected by a mutation in the membrane cofactor protein because the factor is membrane-bound and not circulating[85]. Plasma infusion is similarly ineffective or even contraindicated in patients affected by gain-of-function mutations or in patients affected by a C3 convertase resistant to factor H[63]. Because CFH, CFI, CFB and C3 are produced by the liver, a simultaneous liver-kidney transplantation may be effective and therapeutically useful[86].

In consideration of frequent short-term complications, of the mortality rate of 15% and of the growing experience with eculizumab, an anti-complement drug, a combined liver-kidney transplantation will lose indication[87,88].

**Elimination of the auto-antibodies and/or mutant protein:** The use of plasma exchange has a strong rationale[89], but to date, its efficacy has only been confirmed by single case reports. Three patients with DDD had a beneficial effect from plasma exchange, but they were also treated with immunosuppression[90-92]. However, McCaughan *et al*[93] reported the lack of efficacy of plasma exchange, despite the complete removal of C3NeF. Moreover, in the eculizumab era, the plasma exchange will continue to be used after evaluation of individual patients.

Efficacy of immunosuppression is not yet established.

Treatment with steroids led to a clinical improvement in children affected by C3G treated on the basis of a renal biopsy, revealing signs of acute glomerular inflammation with crescents, but a similar improvement was similarly observed in non-treated patients[94]. The combination of steroids with other immunosuppressants has been reported to have a higher beneficial effect[95-97]. These effects have been principally documented in the aHUS. Treatment with an anti-CD20 monoclonal antibody has been effective in one patient affected by DDD with documented anti-CFB auto-antibodies[59].

Very recently, the beneficial effect of mycophenolate mofetil (MMF) in C3G has been reported in a randomized Spanish study[98]. However, due to the lack of controlled trials, treatment with immunosuppressants should be restricted to patients with proteinuria, progressive loss of glomerular filtration rate (GFR) and those with signs of severe inflammation on renal biopsy[89].

An immunosuppressant-based strategy should also be attempted in patients with C3G associated with monoclonal gammopathy, even if the result of such a treatment differed according to different authors[50,51].

**Inhibition of complement activation:** The most adequate approach to the treatment should be the complement cascade blockade. Eculizumab is a recombinant, fully humanized monoclonal antibody that binds to the C5 complement protein and blocks C5 cleavage[89]. In recent years, eculizumab was highly effective in several kidney diseases, including aHUS and antibody-mediated rejection (ABMR) after renal transplantation[99]. The efficacy of eculizumab in C3Gs to date is only based on the report of single patients, on an open label proof of concept study in 6 patients, and on one ongoing randomized clinical trial (RCT) whose results are unknown to date[100]. Overall, 14 patients affected either by DDD or C3GN treated with eculizumab have been reported. Eight of these patients were described in single case reports and the treatment was successful in seven patients[93,101-107]. In addition to the clinical response, an improvement in renal histology has been observed in patients who underwent a repeated renal biopsy. However, such good results were not confirmed by the proof-of-concept study[108,109]. In this study, a clinical response to eculizumab has been observed in only three patients.

Furthermore, in a recent study, three more patients affected by rapidly progressive C3G have been reported[110]. All these patients responded to eculizumab with an improvement in renal function, a regression of proteinuria and an improvement of glomerular lesions. The phenotypic expression of C3G (DDD *vs* C3GN) does not predict the response to treatment, even if in biomarkers studies, a higher terminal pathway activity in C3GN has been found[111].

Overall, these results revealed disparate results to the treatment and highlight the possibility that complement dysregulation is not always the same in these patients and that in some of the patients, a resistance to C5 cleavage blockade might exist. The unresponsiveness to eculizumab may have different explanations.

Recently, Nishimura *et al*[112] documented that some patients affected by paroxysmal nocturnal hemoglobinuria (PNH) had a missense mutation at arginine 885 at the level of the *C5* gene. This mutation caused a resistance to C5 cleavage by eculizumab.

In addition, patients affected by C3G, after eculizumab administration, may have a persistent fluid phase C3 convertase activity in the absence of terminal complement activity, which has been documented in a patient with C3G caused by a hybrid CFHR2/CHFR5 protein[64]. In this patient, after eculizumab administration, a block of C5 cleavage and sMAC generation has been obtained, but the hyperfunctioning C3 convertase remained active. Consequently, patients with a C3 convertase dysregulation greater than C5 dysregulation should not be treated with C5 blockade[113]. Moreover, has been documented that this block might aggravate the C3 convertase activity *via* a feed-back mechanism. Consequently, patients affected by C3G with a prevailing C3 convertase activity should be treated with drugs inhibiting C3 convertase. Blocking the complement AP at the C3 level might be essential in several patients affected by C3G, but the usefulness of such a blockade should be weighed against potential drawbacks as the block of C3b with its critical role in innate immunity.

To date, there are essentially 3 drugs aimed to exert a blockade at the C3 level. The compstatin analog Cp40 was documented to be effective in inhibiting complement dysregulation *in vitro* in C3G[114]. Compstatin binds to C3 and C3b, preventing the complement dysregulation caused by genetic mutations or by auto-antibodies. To date, compstatin is used in trials for macular degeneration and PNH. Similarly, a monoclonal antibody, which inhibits C3 convertase induced by C3NeF by binding to C3b is currently in the preclinical phase[115]. The most advanced drug among the C3 inhibitors is CDX1135, which is also known as TP10 and the soluble complement receptor 1 (sCR1). CR1 is a cell surface glycoprotein expressed on several cells, including immune cells. sCR1 is a protein that can regulate C3 convertase. Under normal conditions, only small quantities of sCR1 are in circulation. Administration of a high quantity of sCR1 in patients undergoing cardiac surgery revealed that this protein is able to exert a complement inhibition effective and safe[116,117]. Recently, at Iowa University, the efficacy of sCR1 has been documented in vitro and in mice affected by C3G[118].

**Renal transplantation:** C3G has a frequent evolution towards ESRD. Renal transplantation has been proposed for ESRD patients affected by C3G. Renal transplantation in such patients has two principal challenges: (1) whether to perform a dual liver-kidney transplantation; and (2) The high recurrence rates of the disease and its treatment.

The question of liver-kidney transplantation has

been previously mentioned above[86-88] and has been documented that in the eculizumab era, the liver-kidney transplantation will lose its relevance.

In the case of the kidney transplant alone the principal challenge is the high recurrence rate. In the case of DDD, the risk of recurrence is over 70%[119], with a high risk of graft loss[120,121]. These data confirmed a retrospective United States study including 75 children affected by DDD[122] and a more recent Irish cohort, including 33 patients affected by DDD[123]. Fewer data have been reported on the recurrence risk of C3GN. The most relevant study has been published by Zand *et al*[124] from the Mayo clinic. They report 21 renal transplant patients affected by C3GN. The recurrence rate was as high as 70% and the graft failure occurred in 50% of the patients. Importantly, all these reports with high recurrence rates also include patients transplanted in the pre anti-complement era. Transplants in patients affected by CFHR nephropathy has been reported in 11 subjects. All transplants were successful, despite the histological recurrence in three patients[125].

The treatment of recurrent disease has not yet been the object of clinical trials. Close monitoring is mandatory following renal transplantation to promptly detect the clinical signs of recurrence. Patients with circulating auto-antibodies might be treated by agents targeting T and B cells, but we should remember that the transplanted patients are already on immunosuppressant drugs.

Anti-complement drugs are promising. McCaughan *et al*[93] described the first report of a transplanted patient affected by recurrent DDD and who was successfully treated by eculizumab. However, the long-term dependence on eculizumab and the long-term safety of the drug remain open questions and the object of future RCTs. Another interesting approach is the use of sCR1, but its use to date has been limited to the native disease and not to its recurrence after transplantation.

**Clinical trials ongoing:** C3G is a rare disease and it is not surprising that ongoing RCTs are scarce. From one perspective the market interest is poor due to the few numbers of patients. However, a wide comprehensive multinational network among centers should be developed to include a significant number of patients for a RCT.

To date, four clinical trials are ongoing on C3G. Two trials aimed to evaluate eculizumab therapy in DDD and C3GN[100,126]. Two other RCTs are evaluating the effect of two different formulations of sCR1 on C3G[127,128].

Other drugs, such as compstatin and monoclonal antibody against C3 convertase, are still in the pre-clinical phase.

## CONCLUSION

The Mayo Clinic/Renal Pathology Society Consensus Conference held in 2015 allowed the elaboration of a new etiology-pathology based classification of the GN, which substitutes for the old morphologic-based classification (1). In addition, before the Consensus Conference, the MPGNs had been the object of new classifications for several years. To date, it is clear that the MPGNs should be distinguished into two principal categories: The immune-complex-mediated MPGN and the complement-dysregulation-mediated MPGN. This finding is principally relevant, not only from a taxonomic perspective, but also from a diagnostic and therapeutic approach. New findings in the complement related pathways and in genetics allowed for an improved understanding and definition of complement related MPGN, in addition to the discovery of new entities, such as C3GN and CFHR5 GN. To date, the MPGNs have a new diagnostic approach with a new network that applies to the immune-complexes related MPGN and complement-related MPGN.

Currently, fewer new drugs are available for the treatment of immune-complexes MPGN.

With the discovery of complement-inhibitor drugs, there has been more progress for the complement related MPGN. However, due to the rarity of the disease, well conducted RCTs are scarce. This finding supports the need to perform more multinational cooperative studies to identify an evidence-based medicine therapeutic approach.

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MINIREVIEWS

# Renal biopsy: Still a landmark for the nephrologist

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## Abstract

Renal biopsy was performed for the first time more than one century ago, but its clinical use was routinely introduced in the 1950s. It is still an essential tool for diagnosis and choice of treatment of several primary or secondary kidney diseases. Moreover, it may help to know the expected time of end stage renal disease. The indications are represented by nephritic and/or nephrotic syndrome and rapidly progressive acute renal failure of unknown origin. Nowadays, it is performed mainly by nephrologists and radiologists using a 14-18 gauges needle with automated spring-loaded biopsy device, under real-time ultrasound guidance. Bleeding is the major primary complication that in rare cases may lead to retroperitoneal haemorrhage and need for surgical intervention and/or death. For this reason, careful evaluation of risks and benefits must be taken into account, and all procedures to minimize the risk of complications must be observed. After biopsy, an observation time of 12-24 h is necessary, whilst a prolonged observation may be needed rarely. In some cases it could be safer to use different techniques to reduce the risk of complications, such as laparoscopic or transjugular renal biopsy in patients with coagulopathy or alternative approaches in obese patients. Despite progress in medicine over the years with the introduction of more advanced molecular biology techniques, renal biopsy is still an irreplaceable tool for nephrologists.

**Key words:** Renal biopsy; Acute kidney injury; Bleeding; Haematuria; Hematoma; Chronic renal failure



**Core tip:** Percutaneous renal biopsy is an irreplaceable tool in the clinical practice of nephrologists to determine diagnosis, prognosis and treatment of several kidney diseases. This procedure is considered safe if it is performed in well-trained centers. Main indications are acute glomerulonephritis and nephrotic syndrome. Since bleeding is the major primary complication, careful evaluation of risks and benefits must be considered. The risk of complications in patients with coagulopathy may be reduced by using laparoscopic or transjugular renal biopsy or alternative approaches in obese patients. Despite progress in medicine over the years, renal biopsy is still an irreplaceable tool for nephrologists.

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## INTRODUCTION

Percutaneous renal biopsy (PRB) is still considered an irreplaceable tool for diagnosis, prognosis and choice of treatment of several primary or secondary kidney diseases. The indications uniformly recognized by most nephrologists are represented by nephritic and/or nephrotic syndrome and unexplained acute or rapidly progressive renal failure[1]. Primary glomerulonephritis are the more common renal disease in renal biopsy registries. Among them IgA nephropathy (IgAN) is the most frequent renal diagnosis. Regarding systemic diseases, systemic lupus erythematosus (SLE) is the most frequent indication for PRB, because this last determines the level of activity and/or chronicity of the lesions and the reversibility of renal lesion as a result of therapy. PRB can also be helpful in vasculitis to assess the severity of the damage and the potential reversibility after therapy. In diabetes the use of PRB is motivated by a relatively recent or very late appearance of proteinuria > 1 g and/or a rapid decline in GFR and/or active urinary sediment, in the absence of other signs of microangiopathy (retinopathy and neuropathy); in fact, in these patients primitive forms of glomerular diseases are frequently reported, superimposed or not to the typical lesions of diabetes. In advanced chronic renal failure, PRB is useful to assess a rescue therapy or to know the causal nephropathy in view of renal transplantation[2].

PRB is also an informative procedure in renal transplantation, both in the postoperative, for the differential diagnosis of acute rejection *vs* other diseases, and in follow-up of organ transplantation for differential diagnosis between recurrence of primary renal disease, development of glomerulonephritis *ex novo*, and acute or chronic rejection (Table 1).

**Table 1 List of Indications for renal biopsy**

| |
|---|
| Nephrotic syndrome |
| Acute kidney injury (when rule out obstruction, and pre-renal causes) |
| Systemic disease with renal dysfunction (in diabetic patients only if it presents with atypical features) |
| Non-nephrotic proteinuria, and in some circumstances isolated microscopic hematuria |
| Unexplained chronic kidney disease |
| Familial renal disease (may avoid biopsy in other family members affected) |
| Renal transplant dysfunction |

## HISTORY

The first renal biopsy of native kidney was performed in 1901 in a surgical procedure for renal decapsulation in the treatment of a Bright's syndrome[3]. The PRB was born in 1944 when Nils Alwall adapted a technique for percutaneous liver biopsy in the kidney, using an aspiration needle technique[4] with a radiographic procedure for the localization of the right kidney and keeping the patient in a sitting position. With this innovative method, he obtained adequate tissue in ten of the thirteen patients[5]. However, this procedure has been for the first time described in the literature by Iversen and Brun[6] in 1951, which also used an aspiration needle and the sitting position but, in contrast to Nils Alwall, they used intravenous pyelography for localization of the right kidney; unfortunately they obtained adequate tissue only in 53% of patients[6]. Given the poor results of this technique, Kark *et al*[7] in 1954 made significant changes including the prone position of the patients with a sandbag placed under the abdomen to reduce the mobility of the kidney and the introduction of a new type of needle, the Franklin-modified Vim-Silverman needle, which trapped the tissue in the needle and then sheared it off, achieving adequate tissue in 96% of patients and no major complications. To localize the lower pole of the kidney they used as landmark the distances between the vertebral spinous processes and the 11$^{th}$ and 12$^{th}$ ribs, and the movement of a finder needle following a deep inspiration[7]. Over the years the technique has been improved more and more, increasing the adequacy of the sample and reducing the risk of complications.

In 1962 the use of radiological images was introduced for the localization of the kidney, later replaced by the ultrasound real-time imaging. Since then this procedure, which was initially performed by nephrologists, has gradually become a prerogative of radiologists. In fact, between 1964 and 1974 the PRB was performed in 95% of cases by nephrologists[8], while in 1980s the number of nephrologists who performed the PRB was gradually reduced in favour of radiologists and in 2011, Lane *et al*[9] showed that radiologists were the main performers of this technique (Figure 1)[10].

A recent european survey stated that in 60% of the centers renal biopsy is performed by nephrologysts, in 30% by radiologists and in 5% by nephrologysts and radiologists[11]. Today, the standard procedure for PRB involves the use of real-time ultrasound and automated spring-loaded biopsy device[12].

## NEEDLE TYPES AND SIZE

There are different types of biopsy needles and the first used was an aspiration needle, subsequently replaced by the cutting Vim-Silverman needle, which trapped the tissue in the needle and then sheared it off. The evolution of the latter is the Tru-Cut needle, which is a manually operated sheathed needle designed for manual capture of high-quality tissue samples with minimal trauma to the patient. Today it is replaced by automatic spring-loaded biopsy guns and semi-automatic biopsy guns with

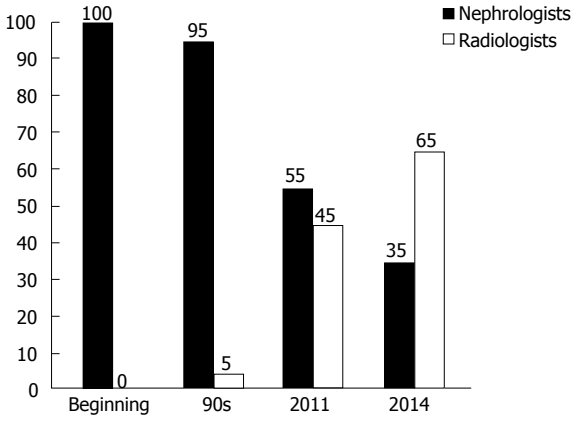


**Figure 1 Rate of performers (nephrologists and radiologists) of renal biopsy along the course of the years[10].**

better and safer performance.

The optimal needle size for native renal biopsies has not been established, but the most used are three: 18 gauge (internal diameter 300-400 μm), 16 gauge (internal diameter 600-700 μm) and 14 gauge (internal diameter 900-1000 μm). The first one is reserved to paediatric patients because the internal diameter of the needle is barely bigger than an adult glomerulus (200-250 pmol/L), while the other two are more appropriate for the adult patients[13,14]. On the other hand, the length of this device is almost the same and is around 20 cm.

## SAMPLE ADEQUACY

The number of glomeruli is the main determinant of the biopsy adequacy but it varies based on the type of glomerular disease. For example in focal disease, such as focal segmental glomerulosclerosis, the diagnosis can be made by identifying even one glomerulus that presents the typical lesions but the probability to make diagnoses is directly proportional to the number of glomeruli[15]. Therefore, in a kidney in which 20% of glomeruli are sclerotic, if a bioptic sample includes five glomeruli the probability to miss affected glomeruli is about 35%. This percentage falls down to 10% if the bioptic sample includes ten glomeruli and to 1% if it includes twenty glomeruli[16,17]. Therefore, the minimum number of glomeruli required to define an adequate bioptic sample is ten, and usually, to get this target at least two different cores are taken which are divided for light microscopy (LM) (placed in formalin or another fixative), immunofluorescence (IF) (placed in transport solution-saline solution- and quickly freezed), and electron microscopy (EM) (fixed in 2%-3% glutaraldehyde or 1%-4% paraformaldehyde)[18].

Actually, the latter is not frequently and widespread performed in the practice of renal biopsy since it is possible to get a diagnosis in most cases with the contribution of the LM and the IF. However, due to the relevance of EM in some specific glomerular diseases, it has been recommended that renal tissue for EM be set aside in each case if EM cannot be performed routinely[19]. As an alternative, IF may be also performed on paraffin sample, using only one core for LM and IF and further reducing the risk of complications resulting from biopsy. The technique is certainly more complicated and needs more time for preparation but provides comparable results with the classic procedure with the exception of complement factors; consequently, it may be used in selected cases and/or in patients with greater bleeding risk.

About the optimal needle size for native renal biopsies, there is not a general consensus to achieve a good compromise between sample adequacy and lower number of complications. In adult patients a 14 or 16 gauge needle seems to be appropriate[20], while in paediatric patients it is better to use 18 gauge needles[21].

## COMPLICATIONS

Even if PRB is considered a safe procedure, it is not without complications (Table 2) that, in very rare cases, may also cause death or require extreme procedures such as nephrectomy[22-24]. For this reason it is always necessary to evaluate the risk/benefit for the patient, inform him/her and obtain a signed consent. Furthermore, complications are divided into major complications that need a treatment or an intervention to stop the problem, and minor complications that spontaneously resolve without intervention or further treatment; in both cases, bleeding is the main consequence of PRB and can occur at different levels: (1) in the collecting duct system, causing micro - gross haematuria which may result in clots formation in the urine (ureter or bladder) with risk of obstructive renal failure; (2) below the kidney capsule, causing subcapsular hematoma formation that in rare cases may lead to the Page kidney, which consists in renal ischemia caused by prolonged compression of the kidney from haemorrhage with resulting arterial hypertension characterized by high renin levels[25]; and (3) in the perinephric space, causing hematoma formation which may be asymptomatic, in the majority of cases, or result into a clinically relevant complication, such as lumbar pain, significant drop in haemoglobin concentration, or need for a blood transfusion.

However, the risk of complications after renal biopsy is not high (Table 3). In fact, in a systematic review and meta-analysis of 34 retrospective and prospective studies including 9474 adult patients who underwent biopsy of the native kidney, using ultrasound real-time imaging and automatic biopsy device, the overall incidence of bleeding complications were: Transient gross haematuria 3.5%, request for transfusion therapy 0.9%, demand on angiographic control of bleeding 0.6%, request for nephrectomy for control of bleeding 0.01% and death 0.02%[26]. Thus, the risk of using invasive procedures to stop bleeding is very rare[27,28]. More frequently we can treat this complication with medical treatment such as administration of endovenous fluid and/or blood products[29]. Moreover in some cases of persistent hemorrhage, before

**Table 2 Types of complications after renal biopsy**

| Minor complications | Major complications |
|---|---|
| Bleeding | Bleeding |
| Asymptomatic haematoma | Hematoma requiring blood transfusion or invasive procedure to stop bleeding |
| Microscopic and gross haematuria | Urinary tract obstruction with or without AKI |
| Anaemia (drop in haemoglobin concentration ⩾ 1 g/dL) | Hypotension related to bleeding |
| Pain (> 12 h) | Nephrectomy |
| Page kidney | Sepsis |
| Perinephric infection | Other organs and/or blood vessels perforation |
| Arteriovenous fistula | Death |

AKI: Acute kidney injury.

**Table 3 List of main studies (> 500 biopsies) reporting minor, major complications and mortality rate after renal biopsy**

| Ref. | Year of publication | No. of biopsies | % Minor complications | % Major complications | % Mortality |
|---|---|---|---|---|---|
| Fenerberg *et al*[24] | 1998 | 1081 | 9.6 | 1.11 | 0.09 |
| Prasad *et al*[28] | 1998 | 1090 | 3 | 0.36 | 0 |
| Preda *et al*[20] | 2003 | 515 | 9.5 | 2.7 | 0 |
| Whittier *et al*[51] | 2004 | 750 | 6.7 | 6.4 | 0.13 |
| Atwell *et al*[44] | 2010 | 5832 | - | 0.7 | 0 |
| Stratta *et al*[29] | 2007 | 1137 | 24.2 | 0.36 | 0 |
| Korbet *et al*[23] | 2014 | 1055 | 8.1 | 6.6 | 0.09 |
| Mai *et al*[21] | 2013 | 934 | 5.9 | 0.86 | 0 |
| Tøndel *et al*[13] | 2012 | 9288 | 1.9 | 0.9 | 0 |
| Prasad *et al*[28] | 2015 | 2138 | 5.4 | 5.1 | 0 |

performing embolization of a pseudoaneurysm or surgery to stop the bleeding, we can resort to off-label drug use such as recombinant activated factor Ⅶ[30].

### *Specific symptoms and signs post-biopsy*

**Lumbar pain:** The pain is an extremely common consequence of PRB and usually occurs at the end of anaesthesia. If necessary it is possible to administer a mild analgesic. Otherwise, the onset of greater pain suggests the development of a major complication and further diagnostic tests must be performed.

**Microscopic haematuria:** It is the most common consequence of this procedure; it is usually asymptomatic[31] and resolves spontaneously over a few days.

**Gross haematuria:** It occurs in 3% of renal biopsies and typically disappears in few hours or days. Occasionally gross haematuria may cause a significant drop in haemoglobin concentration requiring a blood transfusion or, in rare cases, it may result in clots formation with or without obstructive renal failure. On the contrary, persistent haematuria after three days suggests the onset of major complications such as arteriovenous fistula (AVF)[32].

**Acute anaemia:** A decrease of haemoglobin concentration ⩾ 1 g/dL occurs in more than 50% of uncomplicated renal biopsies[33], whereas a fall ⩾ 2 g/dL occurs in 10% of uncomplicated cases and is consequently associated with increased risk of complications[34].

**Perinephric hematoma:** The presence of asymptomatic hematoma is frequently detected during a renal ultrasound after biopsy and does not constitute *per se* a complication. Prospective studies showed that perinephric hematoma is detectable in 90% of patients 24-72 h after the procedure, while this percentage drops to 15% immediately after the biopsy. Most of the perinephric hematomas are small, asymptomatic and they resolve spontaneously in few months; only in 2% of cases they may cause a clinically relevant complication such as lumbar pain, a decrease in haemoglobin concentration, or the need for blood transfusion. However, the absence of hematoma at 1 h was highly predictive of an uncomplicated course[35].

Waldo *et al*[36] showed that patients which did not present perinephric hematoma one hour after biopsy did not develop major complications in 95% of cases, while the presence of hematoma was predictive for major complications in 43%. Therefore, the routine use of ultrasound at 1 h after PRB may have a role in determining an uncomplicated course[36].

**AVF:** It is not a frequent complication and is due to trauma of the wall of blood vessels; it is clinically asymptomatic and resolves spontaneously in most cases[37]. In rare cases AVF can cause the development of an aneurysm, which may manifest clinically with high

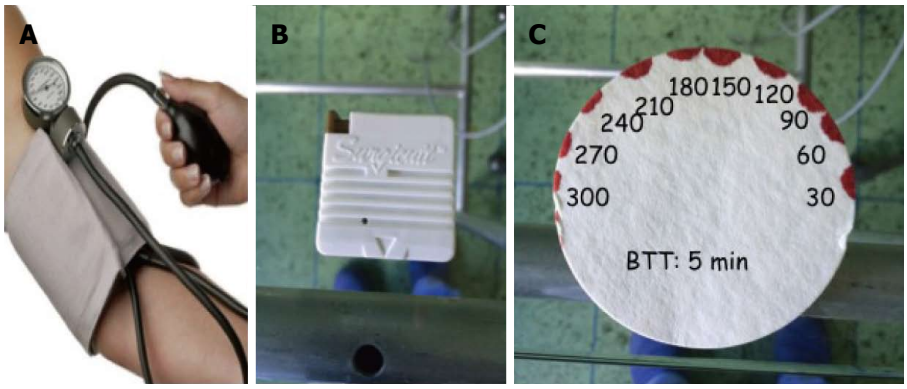


**Figure 2 Bleeding time procedure.** A: Place the sphygmomanometer on the upper arm and inflate to 40 mmHg; B: Make a small cut on the lower arm with automatic standard device; C: Blotting paper is used to draw off the blood every 30 s (normal range 3-7 min).

blood pressure, heart failure, and kidney failure. Important signs that suggest this complication are the persistence of gross haematuria, the presence of abdominal bruit and palpable trill[38,39] but diagnosis confirmation requires Doppler ultrasound or magnetic resonance imaging, or angiography. The treatment of symptomatic cases is based on superselective transcatheter arterial embolization or, in rare cases, surgery[40].

## CONTRAINDICATIONS AND RISK FACTORS

Contraindications to renal biopsy and risk factors must be taken into account to minimize the risk of complications.

The presence of intravascular coagulopathy, polycystic kidneys, obstruction of the urinary tract, hydronephrosis, infections of the upper urinary tract are regarded as absolute contraindications. Otherwise, there are some conditions, which require caution, considered as relative contraindications, such as compromised cardiopulmonary function or hemodynamic instability, severe obesity, inability of the patient to cooperate, solitary kidney, advanced age, severe hypertension (> 160/95 mmHg), and renal failure[41]. The last one causes functional alterations of coagulation factors as the von Willebrand factor (vWF) and the Factor Ⅷ, abnormalities in platelet membrane, accumulation of uremic toxins that inhibit platelet aggregation, high levels of prostacyclin and nitric oxide which are factors that reduce platelet aggregation. Another element that often contributes to increase the risk of bleeding in renal failure is the presence of anaemia. Other diseases associated with greater risk of bleeding are those with arteriolar involvement as SLE, vasculitis, scleroderma, amyloidosis and advanced diabetic nephropathy because they interfere with the first mechanism of haemostasis, known as the vascular phase, reducing the arteriolar contraction.

## PROCEDURES PRE-BIOPSY

Before performing the PRB it is very important to follow some recommendations to minimize the risk of complications. Renal ultrasound is essential to evaluate the presence of anatomical abnormalities of the kidney (presence of multiple cysts, hydronephrosis, solitary kidney) that may represent a risk factor for the development of complications.

Laboratory tests may reveal the potential presence of coagulopathy. To totally assess the steps of haemostasis it is useful to use the bleeding time that evaluates the time of platelet aggregation (Figure 2). In case of advanced renal failure and/or prolonged bleeding time, the administration of desmopressin acetate - DDAVP (0.3 μg/kg), estrogen and cryoprecipitate has shown a reduction of the bleeding risk[42,43].

Antiplatelet agents and oral anticoagulants have to be withdrawn at least one week before renal biopsy[44], the last ones until normalization of INR, and replaced with low molecular weight heparin (LMWH). Other drugs that may cause alterations in coagulation are the non-steroidal anti-inflammatory drugs (NSAIDs), which should be not taken for at least 5 d before PRB.

## ALTERNATIVE APPROACHES FOR RENAL BIOPSY

In some cases, PRB may be contraindicated because of bleeding diatheses or habitus of the patients such as obesity. In these circumstances we can perform renal biopsy with alternative methods such as under CT guidance[45] or with laparoscopic[46] and transjugular approach[47]. These techniques may have some limits. CT guidance, for example, does not assess any possible movements of the kidney related to breathing, laparoscopic biopsy requires general anaesthesia and transjugular biopsy seems to be associated with a lower diagnostic power due to the need to pass through the medulla first[48].

In obese patients a new approach of PRB under real-time ultrasound guidance has been proposed with the patient in supine antero-lateral position (SALP). Gesualdo *et al*[49] reported a case series of 110 patients undergoing PRB, divided into two groups: Low risk group (90 patients) if the body mass index (BMI) was ⩽ 30 in the absence of respiratory disorders and high risk group (20 patients) if BMI was > 30 with breathing problems. The first group underwent classical PRB in prone position and the other group in SALP, demonstrating, at the end of the study, that there were no substantial differences about adequacy samples and patients safety[49]. Moreover, an open renal biopsy may be performed when uncorrectable

contraindications are present. Nomoto *et al*[50] reported 931 cases of open kidney biopsies concluding that this is a safe procedure with 100% of sample adequacy but an important limitation of this technique is the use of general anesthesia.

## PERIOD OF OBSERVATION

After biopsy, the patient must be at rest for at least 6-8 h in the supine position. Blood pressure should be monitored frequently, and urine must be checked to evaluate the presence of gross haematuria. If there are no signs of bleeding within 6 h, the patient may sit up, because most of complications occur within 6-8 h. However, since some complications may also occur later, the ideal observation time should be continued for 24 h. In a case series of 750 biopsies of native kidney it was reported that 67% of major complications appeared within the first 8 h, suggesting that observation for 24 h is safer in renal biopsy[51].

## CONCLUSION

PRB is a safe procedure and the risk of development of major complications is very rare. Instead, the minor consequences due to the procedure occour more frequently. These are micro- and/or gross haematuria, drop in hemoglobin concentration > 1 g/dL, development of asymptomatic perinephric hematoma. All these minor adverse events can be more safely managed and do not bring particular complications to the patient. It is mandatory to identify risk factors for bleeding such as anaemia, prolonged bleeding time or advanced renal failure, severe arterial hypertension and correct them when possible; where this is not possible, it is recommended to postpone the procedure.

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# Management of nocturnal enuresis - myths and facts

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## Abstract

Nocturnal enuresis often causes considerable distress or functional impairment to patient and their parents necessitating a multidisciplinary approach from paediatrician, paediatric nephrologist, urologists and psychiatrist. Mechanisms of monosymptomatic nocturnal enuresis are mainly nocturnal polyuria, bladder overactivity and failure to awaken from sleep in response to bladder sensations. Goal oriented and etiology wise treatment includes simple behavioral intervention, conditioning alarm regimen and pharmacotherapy with desmopressin, imipramine and anticholinergic drugs. Symptoms often recurs requiring change over or combination of different modes of treatment.

**Key words:** Nocturnal enuresis; Monosymptomatic; Conditioning alarm; Desmopressin; Imipramine



**Core tip:** Nocturnal enuresis often causes considerable distress to patient and their parents' lifestyle necessitating a multidisciplinary management. Simple behavioral interventions, conditioning alarm regimen and pharmacotherapy as desmopressin, imipramine and anticholinergic drugs are the mainstay of therapy used as per underlying etiology or parents' concern. Therapy should be structured and goal directed to reduce recurrence.

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## INTRODUCTION

Enuresis though often conceived as a simple problem can have multiple hidden etiologies necessitating a multidisciplinary approach involving paediatrician, paediatric nephrologist, urologists and child and adolescent psychiatrist. The complexity in both assessment and treatment underscores the need for practice parameters

**Table 1 Lower urinary tract symptoms**

| |
|---|
| Consistently increased (≥ 8 times/d) or decreased (≤ 3 times/d) voiding frequency |
| Daytime incontinence |
| Urgency |
| Hesitancy |
| Straining (application of abdominal pressure to initiate and maintain voiding) |
| A weak stream |
| Intermittency (micturition occurs in several discrete spurts) |
| Holding maneuvers (strategies used to postpone voiding) |
| A feeling of incomplete emptying |
| Post-micturition dribbling |
| Genital or lower urinary tract pain |

for clinicians confronting this problem.

## DEFINITIONS

As per DSM-IV-TR, enuresis is defined as repeated voiding of urine into the bed or clothes at least twice per week for at least three consecutive months in a child who is ≥ 5 years of age[1]. A child may also be considered to be enuretic if the frequency or duration is less, but there is associated distress or functional impairment. As per International Children's Continence Society (ICCS), enuresis can be defined as urinary incontinence while asleep in a child aged at least 5 years[2]. The DSM-Ⅲ and ICD-10 define a bed-wetting frequency of twice per month in the past 3 mo for children ages 5 and 6 years and once per month in the past 3 mo for children ages 7 years or older. The DSM-IV-TR includes voluntary as well as involuntary voiding, although most studies exclude children who voluntarily or intentionally wet their bed or clothes. Nocturnal enuresis refers to voiding during sleep; diurnal enuresis defines wetting while awake.

## TYPES

Enuresis may be of monosymptomatic or non-monosymptomatic forms.

Monosymptomatic enuresis (MNE) denotes enuresis in children without any other lower urinary tract symptoms and without a history of bladder dysfunction[2]. Non-monosymptomatic (NMNE) enuresis is defined as enuresis in children with other lower urinary tract symptoms (Table 1). It can also be classified as primary enuresis occurring in children who have never been consistently dry throughout the night, or secondary enuresis which refers to the resumption of wetting after at least 6 mo of dryness[3].

## EPIDEMIOLOGY

The reported prevalence of enuresis at different ages varies considerably because of inconsistencies in its definition as stated earlier, differences in the method of data collection, and differences in the characteristics of the population sampled. Nocturnal incontinence occurs in 12% to 25% of 4-year-old children, 7% to 10% of 8-year-old children, and 2% to 3% of 12-year-old children[4]. It may be problematic even in late teenage years (1% to 3%)[5] and if untreated enuresis (especially if severe) can persist indefinitely with prevalence rates of 2%-3% in adulthood[6,7]. Primary enuresis is twice as common as secondary enuresis. Enuresis seems to be more common among boys (2:1) in whom the problem is often more difficult to treat[8,9]. Enuresis is more common at all ages in lower socioeconomic groups and in institutionalized children. Majority of children have primary nocturnal enuresis whereas children with secondary enuresis may have precipitating factor such as an unusually stressful event (*e.g.*, parental divorce, birth of a sibling, school trauma and sexual abuse). The spontaneous cure rate of night time enuresis is 14% to 16% annually[10].

## ETIOLOGY

Factors that are believed to contribute to enuresis include genetics, sleep disturbances, maturational delay and abnormal secretion of antidiuretic hormone (ADH, vasopressin). Psychological and behavioral abnormalities although common are likely to be a result of enuresis rather than the cause.

### *Genetics*

Bakwin showed that compared with a 15% incidence of enuresis in children from non-enuretic families, 44% and 77% of children were enuretic when one or both parents, respectively, were themselves enuretic. Scandinavian linkage studies depicted a locus for enuresis on chromosome 13 (ENUR 1) and another (ENUR 2) on chromosome 12[11,12].

### *Sleep aspects*

Whether sleep disturbances are a result of the enuresis or contributes to the pathogenesis of enuresis is still debatable. Attempts at arousal were more often successful in control subjects than in boys with enuresis (40% *vs* 9%)[13]. In contrast another sleep study found that children with severe enuresis were actually "light sleeper" but they did not wake before voiding[14]. The arousal centre may be suppressed in these children. Persistently overactive bladder may lead to the abnormal arousal response just like the analogy of someone constantly knocking at the door leading to one either ignoring the knock or even installing an extra lock. Enuresis has been associated with snoring or sleep apneas due to adenotonsillar hypertrophy. This may be due to paradoxical rising of the arousal threshold due to constant stimuli from the obstructed airways or polyuria secondary to increased anti natriuretic peptide due to persistent negative intra-thoracic pressure found in sleep apnea syndrome.

### *Maturational delay*

Since most cases of MNE resolves spontaneously a delayed maturation of a normal developmental process has been explored. Increased incidence of delayed language and slowed motor performances has been identified in some studies among children with enuresis[15]. Urodynamic and EEG findings have shown progressive maturation in bladder stability along with EEG changes suggesting increased central nervous system recognition of bladder fullness and the ultimate ability to suppress the onset of bladder contraction. Bladder capacity at birth is only around 60 mL and thereafter increases with age[16]. Children with nocturnal enuresis have been noted to have a smaller bladder capacity (functional rather than anatomical) even when there are no day time concerns[17]. There are reports of lower average height and lower mean bone age and late sexual maturation in enuretic than in non-enuretic children and adolescent. There is a greater incidence of enuresis in children who were delayed in the attainment of motor and language milestones as well.

### *Nocturnal polyuria*

Increased urinary output overnight might also play an important role in MNE[18]. The cause may include increased fluid intake before bedtime, reduced response to antidiuretic hormone, and or decreased secretion of ADH.

### *Role of ADH*

Despite the utility of desmopressin in the treatment of MNE the relationship between ADH secretion and night time urinary output remains controversial.

Initial studies did suggest presence of a blunted response to vasopressin in enuretic children compared with age-matched controls but subsequent studies failed to reproduce this observation[19].

Some studies have also demonstrated decreased nocturnal secretion of ADH but whether this is primary or secondary to the small bladder capacity (ADH secretion is thought to be stimulated with bladder distension) is not clear[20].

Additionally it needs to be emphasized that abnormalities in ADH secretion does not explain as to why these children do not wake to void.

### *Psychosocial factors*

Psychiatric disorders in children with enuresis are higher than the rate found in non-enuretic groups but the relationship may be of etiologic relevance or it may be coincidental or occurring in response to the symptom of enuresis[9]. Children with enuresis had 2.88 times increased odds (95%CI: 1.26-6.57) of having attention deficit hyperactivity disorder (ADHD) as compared with those without enuresis[21]. It has been suggested that both enuresis and ADHD might be related to delays in central nervous system maturation[22]. Enuresis has sometimes been described as a masturbatory equivalent, an expression of bisexuality, or the somatic expression of a defect in body image.

### *Adverse event to medications*

Enuresis may rarely results as a side effect of a medication such as lithium, valproic acid, clozapine and theophylline (secondary enuresis).

## MECHANISM

The pathophysiology of enuresis is complex, involving the central nervous system (several neurotransmitters and receptors), circadian rhythm (sleep and diuresis), and bladder function derangements. Urinary continence is obtained in three sequential steps: Enlargement of the bladder capacity, voluntary control of the sphincter muscles, and voluntary control of the micturition reflex.

There are three commonly proposed mechanisms to bedwetting (Figure 1)[23,24].

The locus coeruleus (LC), a noradrenergic neuron group in the upper pons is crucial for arousal from sleep and overlaps both functionally and anatomically with the pontine micturition centre, which coordinates the micturition reflex. The LC also has axonal connections with the hypothalamic cells that produce vasopressin[25-27]. Hence disturbances in this region of brainstem might be the missing link to a unifying pathogenic mechanism.

## EVALUATION

Evaluation of a child with enuresis consists of detailed history, focused examination and appropriate investigations.

### *History*

Detailed history is the key to the treatment success of a child suffering from enuresis. In every instance, both the parents and the child should be interviewed, and sensitivity to the emotional consequences of the symptoms should be high. Special focus in history should include: (1) Daytime wetting/urgency/holding maneuvers/weak or interrupted urinary stream including dribbling or straining; (2) Primary or secondary enuresis; (3) Frequency and pattern of nocturnal enuresis (including number of wet nights per week or month, number of episodes per night, time of episodes, approximate volume of each episode); (4) Daily fluid intake and urine output diary. (This will identify whether the child drinks adequately as well as whether the majority of fluid intake happens in late afternoon/evening, daytime urinary frequency, presence of polyuria - which might indicate other underlying cause such as diabetes, kidney disease or psychogenic polydipsia); (5) Stool history (including history of constipation/fecal soiling/encoperesis); (6) Any relevant medical history (*e.g.*, review of history of sleep apnea, sickle cell disease or trait, diabetes, recurrent urinary tract infection, gait/neurological abnormalities); (7) Details of any previous interventions for enuresis; (8) Family history of nocturnal enuresis; (9) Social history (may be important in secondary enuresis);

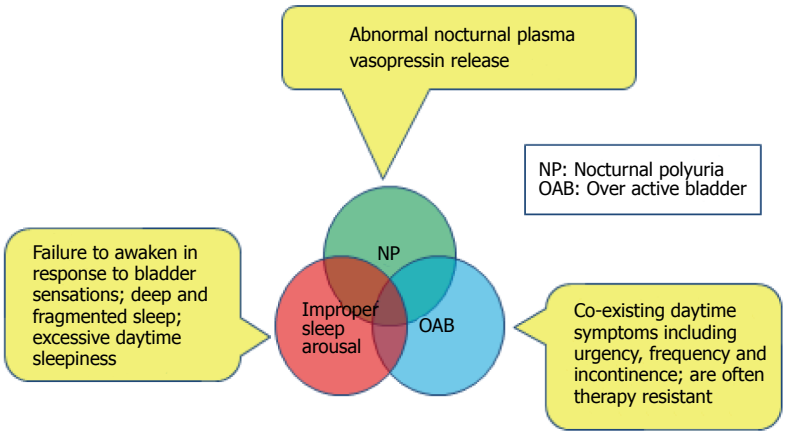


**Figure 1 Mechanisms to bedwetting: Three commonly proposed mechanisms often overlap each other.**

(10) Importantly effort should be made to understand how the problem has affected the child and family and the degree of motivation in both the child and family; (11) Behavioral history including behavior screening questionnaire; and (12) The sleeping arrangements for the child at home should be explored.

A voiding diary is helpful in not only identifying any underlying bladder dysfunction such as day time frequency but also in establishing a baseline record of the enuresis pattern. This may serve as standard against which the success of subsequent interventions can be gauged. Not infrequently, this baseline monitoring itself is associated with a dramatic improvement.

### *Physical examination*

Although in most of the cases (particularly in children with MNE) physical examination is usually normal, detailed physical examination is still important to ensure any other underlying etiology is not being missed. A quick but focused physical examination in children with enuresis should include: (1) Growth: Poor growth may indicate an underlying renal problem and should prompt further examination attempting to identify any other renal disorder related signs such as hypertension; (2) Adenotonsillar hypertrophy or other signs of sleep apnea: Rarely they may be the underlying cause for enuresis; (3) Abdominal palpation: Will help in identifying fecal mass (severe constipation/encoparesis) or distended bladder (bladder outlet obstruction); (4) Perianal excoriation or vulvovaginitis which may indicate pinworm infection; (5) Detailed examination of lumbosacral spine as well as neurologic examination of perineum and lower limbs will aid in identifying any occult spinal cord abnormalities; and (6) Detection of wetness in the undergarments may be a sign of daytime incontinence.

### *Appropriate investigations*

Investigations are usually minimally required in children with MNE. Cayan reported that the findings of ultrasonography and uroflowmetry were no different in children with nocturnal enuresis than in children without the condition[28]. So performing more than a urinalysis and culture for children with nocturnal enuresis would neither be cost effective nor helpful to the child. More invasive tests are indicated only in NMNE.

**Urinalysis:** This can aid in ruling out ketoacidosis, diabetes insipidus, water intoxication, and/or occult urinary tract infection[29]. First-morning specific gravity may be helpful in predicting who will respond to desmopressin treatment.

**Imaging:** Ultrasonography may be useful in NMNE for estimating bladder capacity, post-void residual volume, and bladder wall thickness. Voiding cystourethrogram can be useful in children with significant daytime complaints or history of recurrent urinary tract infection. Abdominal radiograph although rarely used for determining the presence and/or extent of stool retention is also helpful in convincing the parents about the severity of the constipation[30]. Neuroimaging such as magnetic resonance imaging of the spine will be needed if the lumbosacral/perianal/lower limb neurological examination demonstrates any abnormality[31].

**Urodynamic studies:** Urodynamic studies are limited to children with suspected bladder dysfunction as per history/examination and or ultrasound or voiding cystourethrogram results.

Frequently, the psychological and developmental damage may actually be more significant and devastating to the child than the symptom of enuresis itself so many a times psychological evaluation may be needed.

## DIFFERENTIAL DIAGNOSIS

Although with detailed history and examination the diagnosis is not very difficult but following underlying conditions should not be overlooked: (1) Underlying medical conditions resulting in polyuria such as sickle cell disease, diabetes mellitus, diabetes insipidus, *etc.*; (2) Severe constipation/encoperesis; (3) Bladder bowel dysfunction; (4) Spinal dysraphism; (5) Chronic kidney disease such as nephronopthisis; (6) Pinworms; (7) Psychogenic polydipsia; and (8) Upper airway tract obstruction.

## TREATMENT

Bed wetting while asleep is considered normal at least

till 5 years. Even subsequently need for intervention is often not a medical decision being influenced primarily by the family and the child's perception towards enuresis.

### *Evaluating the impact on the child and family*

In children aged ⩾ 5 years, enuresis is considered abnormal. Reasons for proactive management can be manifold including the distress caused to child and family, difficulty of "sleeping over" on holiday or at friends' houses, social withdrawal, reduced self-esteem, and potential disturbance of the child's and the parents' sleep architecture that may have an impact on daytime functioning and health[32]. Additional reasons include the risk that some parents may be intolerant of their child's wetting and the significant inconvenience and costs associated with frequent laundering of bedsheets and clothing[33]. In primary care, "trial and error" treatment for enuresis is often the rule rather than the exception; this approach is a waste of time and money and increases frustration among families and doctors. It may also have an adverse psychological effect on the child.

Prior to initiation of any management it is important to understand the prime concern of the family as well as their expectation. The age at which enuresis is considered to be a "problem" varies depending upon the family. If both parents wet the bed until late childhood, they may not be concerned that their seven-year-old wets the bed. In contrast, parents may be concerned about a four-year-old who wets if he has a three-year-old sibling who is already dry. Often the family may not want any active intervention once they understand the self resolving nature of the problem as well as the usual absence of any identifiable underlying physical anomaly. Sometimes the family wants a quick solution (maybe for a planned travel or sleep over) or often is aiming for a long term cure. Therapy should be goal-oriented, and follow-up should be consistent.

### *Goals of treatment*

The goals of interventions for nocturnal enuresis include[34]: (1) To stay dry on particular occasions (*e.g.*, sleep over); (2) To reduce the number of wet nights; (3) To reduce the impact of enuresis on the child and family; and (4) To avoid recurrence.

Historically nocturnal eneuresis management as per Glicklich's review explored fascinating "treatments" as cautery of sacral nerves, penile ligation, inflated vaginal balloons to compress the bladder neck, and electric shocks to the genitalia. Structured approach has been show to be beneficial and is professed to be the approach of choice. History, physical examination and or laboratory tests give clues as to the management plans. Daytime wetting, abnormal voiding (unusual posturing, discomfort, straining, or a poor urine stream), a history of urinary tract infections or evidence of infection on urinalysis or culture, and genital abnormalities are indications for nephro-urologic referral and subsequent treatment plan is influenced by any identified underlying aetiology. In case of coexisting constipation-disimpaction and establishment of a healthy bowel regimen leads to better control of enuresis. Snoring and enlarged tonsils or adenoids may signal sleep apnea and surgical correction of upper airway obstruction may result in improvement or cure of enuresis.

### *Step 1*

**Simple interventions:** Initial interventions are usually restricted to educational and simple behavioral interventions. A number of common sense approaches (Table 2) to enuresis have evolved over time and despite lack of evidence they can be considered supportive for uncomplicated MNE.

**Behavioral interventions:** Behavioral interventions for treating bedwetting are defined as interventions that require a behavior or action by the child that promotes night dryness and includes strategies which reward that behavior. These include: (1) Simple behavioral interventions - behaviors or actions that can be achieved by the child without great effort; (2) Complex behavioural interventions - multiple behavioural interventions which require greater effort by the child and parents to achieve, including enuresis alarm therapy.

Simple behavioral interventions are often used as a first attempt to improve nocturnal enuresis and include reward systems such as star charts given for dry nights, lifting or waking the children at night to urinate, retention control training to enlarge bladder capacity (bladder training) and fluid restriction.

Awakening the child to void during the night (to pre-empt the symptom). Generally, given the enuretic child's sound sleeping ability, this does not lead to significant sleep disruption.

Lifting: Involves taking the child to the toilet during the night usually before the time that bedwetting is expected, without necessarily waking the child.

Waking: Involves waking the child to allow him/her to get up and urinate.

Neither waking nor lifting children and young people will promote long-term dryness but can be used in the short-term management of nocturnal enuresis.

Reward systems (*e.g.*, star charts): The child might receive a star for every dry night, and a reward after a preset number of stars have been earned[35].

Bladder-stretching exercises to increase functional bladder capacity have been used without consistent evidence of effectiveness. The effort not to void despite considerable urgency is unpleasant for both the child and the family.

Retention control training: Attempting to increase the functional bladder capacity by delaying urination for extended periods of time during the day.

Stop-start training: Teaching children to interrupt their stream of urine in order to strengthen their pelvic

**Table 2 Common sense approaches for the management of uncomplicated monosymptomatic nocturnal enuresis[39,40]**

| What to do | How to do | How it works |
|---|---|---|
| Educate parents about | High prevalence of enuresis | Reduce their guilt |
| | Relatively high spontaneous cure rate | Encourage hope |
| | Non-volitional nature of the symptom | Avoid a punitive response or the development of a control struggle |
| Encourage child | Keeping of a journal | Raises awareness in the child |
| | Keeping a dry bed chart | |
| | Changing the wet bed | |
| Maintain voiding diary record | Daytime diary used to: Measurement of maximum voiding volume (excluding the first morning void); over a minimum of 3-4 d for accuracy; measurement on weekends or school holidays are ideal | Assess the child's bladder capacity |
| | Bedwetting diary completed for seven consecutive days/nights | Assess for the presence of nocturnal polyuria |
| Fluid intake regulation | Decrease fluids especially caffeinated beverages, before bedtime. | Decrease nocturnal urine production |
| | Ensuring adequate fluid consumption in the morning and afternoon and avoiding excessive fluid during evening | |

floor muscles.

The impact of bedwetting can be reduced by using bed protection and washable/disposable products; using room deodorizers; thoroughly washing the child before dressing; and using emollients to prevent chafing.

Urotherapy is a commonly used terminology and usually includes education on normal bladder function, regular voiding habits and voiding posture, lifestyle advice regarding fluid intake and prevention of constipation and instruction on the use of bladder diaries or frequency-volume charts[36]. It encompasses various methods of pelvic floor muscle training, behavioral modification, neuromodulation and catheterization. The first-line treatment of daytime incontinence in childhood is basic urotherapy, *i.e.*, advice regarding fluid intake and regular voiding habits. The same advice is routinely given to enuretic children as well. This is not illogical, given the role of detrusor over activity in enuresis, but to date evidence for the efficacy of this approach is weak. However, urotherapy is certainly not harmful and alleviates concomitant daytime symptoms.

Simple behavioural methods in twelve randomised controlled trials including a Cochrane review found it to be superior to no active treatment but appear to be inferior to enuresis alarm therapy and some drug therapy (such as imipramine and amitriptyline).

Despite anecdotal reports, there is no empirical evidence to suggest efficacy of hypnotherapy, dietary manipulation, acupuncture, chiropractic treatment and psychotherapy and desensitization to allergens[37].

### *Step 2*

**Active interventions (enuresis alarms/pharmacotherapy):** Active interventions are usually planned if simple strategies as discussed above fail to yield positive results even after 3 to 6 mo. These interventions are usually based on recommendations of ICCS standardization document on MNE which have been reviewed and endorsed by committees representing the American Academy of Pediatrics, European Society for Paediatric Urology, European Society for Paediatric Nephrology, and the ICCS. Two first-line treatment options are suggested - desmopressin and enuresis alarm[38]. The choice of initial treatment may be based on the parents' and child's preference, their motivations, the physician's experience and local resources.

Information from diaries may identify one of four subtypes of MNE and allow further fine-tuning of treatment (Figure 2).

**Indications for referral**: MNE usually can be managed effectively by the primary care provider. However, children with refractory nocturnal enuresis may benefit from referral to a healthcare professional who specializes in the management of recurrent or refractory enuresis (*e.g.*, developmental-behavioral pediatrician or urologist if structural or anatomic abnormalities are suspected). Additional indications for referral include non-monosymptomatic enuresis; developmental, attention or learning difficulties; behavioral or emotional problems; and known or suspected physical or neurologic problems.

**Conditioning regime:** Conditioning awakening to the sensation of a full bladder is the most benign and successful of the generic treatments of enuresis since its description in 1938. A careful meta-analysis of decades of conditioning studies has shown an initial success rate (defined as a reduction to less than one wet night per month) of approximately 66%, with more than half the subjects experiencing long-term success[39]. The few existing studies that compare conditioning with pharmacologic treatments have generally shown conditioning to be significantly more effective than imipramine[40] and desmopressin (DDAVP)[41].

### *Enuresis alarm*

Enuresis alarms is the most commonly prescribed conditioning regime which has a level 1, grade A International Consultation on Incontinence (ICI) recommendation. Portable transistorized alarms that the child wears on the body have replaced the old bell-and-pad type, but the principle is the same. The first drops of urine moisten the fabric separating two electrodes, thereby completing the circuit and setting off the alarm that the child is wearing.

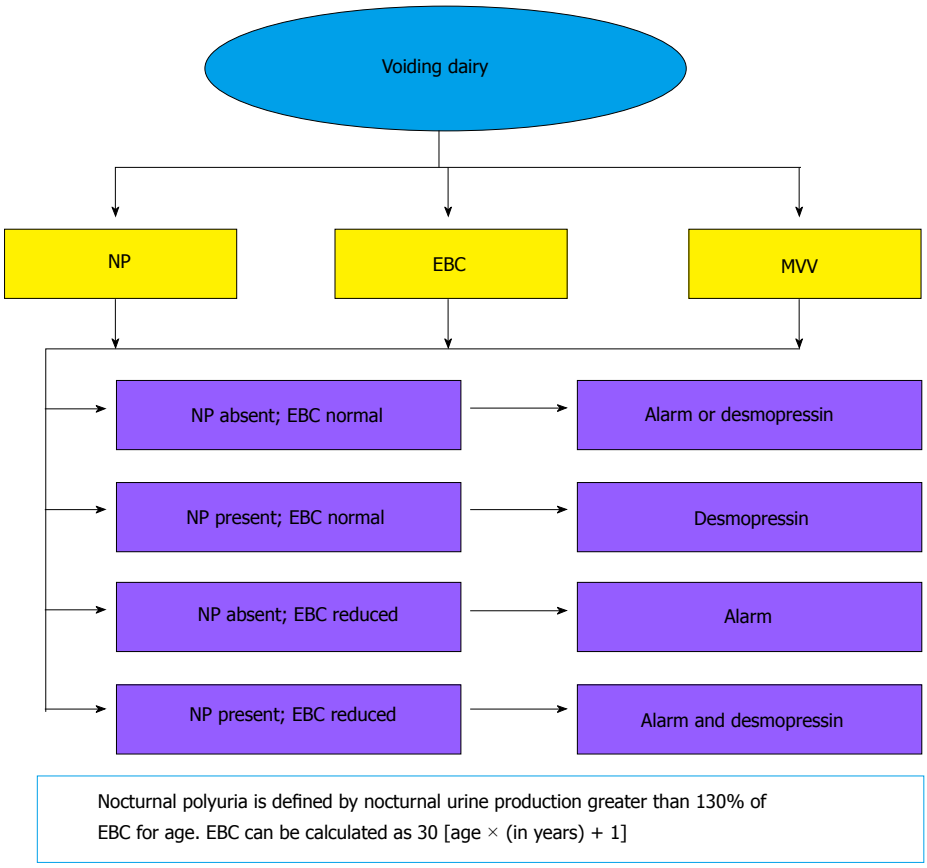


**Figure 2 First line management of monosymptomatic nocturnal enuresis as per voiding dairy[45].** NP: Nocturnal polyuria; EBC: Expected bladder capacity; MVV: Maximum voiding volume.

Initially if children do not wake with the noise or vibration, it is important for their parents to wake them. Gradually the child awakens earlier and earlier in the course of the enuretic episode and the wet spot diminishes in size until the sensation of bladder fullness causes the child to awaken before wetting. Response is not immediate and treatment should be continued for 2-3 mo or until the child is dry for 14 consecutive nights (whichever comes first). Success is followed by over-learning (*e.g.*, extra drinks are given at bedtime to cause additional stress to the detrusor muscles in the bladder. Alarm treatment is then continued until 14 consecutive dry nights are once again achieved) and intermittent reinforcement in which the child uses the alarm every other day before discontinuing it. Lack of parental help to awaken the child to finish voiding in the toilet is a major reason for failure of the conditioning treatment.

Enuresis alarm should not be tried if: (1) The child wets the bed only once or twice per week; (2) The child or parents do not seem to be enthusiastic about the enuresis alarm; (3) Rapid or short-term improvement seems to be the goal for the parents; and (4) The parents seem to express negative feelings/blame their child for wetting the bed.

Lack of success with the approach in the past or a relapse after previous success does not preclude successful subsequent treatment with a conditioning device. Throughout the behavioral treatment, rewarding the success with a sticker chart and reinforcing positive change is critical to maintaining the child's investment in the process. Enuresis alarms are by far the most effective means of long term control as well as preventing relapses. In a meta-analysis of 56 randomized trials (3257 children), sixty-six percent of children became dry for 14 consecutive nights during alarm use *vs* only 4% in the no-treatment control group [relative risk (RR) for treatment failure 0.38, 95%CI: 0.33-0.45]. Additionally nearly a half of children remained dry even after stopping the treatment, compared with almost none in the no-treatment group (45% *vs* 1%, RR for relapse 0.56, 95%CI: 0.46-0.680)[42].

**Pharmacotherapy:** Two medications, DDAVP and imipramine have proven efficacy in the treatment of enuresis.

Desmopressin is a synthetic analogue of the ADH vasopressin which has been used to treat central diabetes insipidus, bleeding disorders such as von Willebrand disease and primary nocturnal enuresis. It decreases urine production at night when taken at bedtime. Desmopressin has a level 1, grade A recommendation from the ICI in 2009[43]. It is administered orally in 0.2 mg tablets in doses of 0.2 to 0.6 mg nightly or, less commonly, intra-nasally as a spray in doses of 10 to 40 μg (one to four sprays) nightly; the lowest effective dose is determined empirically with each child. Due to variable absorption and risk of over dosage nasal sprays are

usually not advocated. Desmopressin is also available as a fast-melting oral lyophilisate (melt; dosage, 120-360 μg). As this form does not require extra water to take this medication, it has become popular particularly for children under 12 years medication should be taken 1 h before the last void before bedtime to allow timely enhanced concentration of urine to occur. Fluid intake should be reduced from 1 h before desmopressin administration and for 8 h subsequently to encourage optimal concentrating capacity and treatment response, as well as to reduce the risk of hyponatremia/water intoxication. Desmopressin is primarily utilized as an alternative to enuresis alarms for children and families who seek rapid or short-term improvement of enuresis; where enuresis alarms have failed or have been refused by family or are unlikely to succeed because of family dynamics. The initial duration of treatment should be for 2-6 wk, to ascertain its anti-enuretic effect. If a sufficient degree of improvement is experienced, then treatment can be continued for an additional 3 mo - where appropriate. Structured withdrawal of medication may reduce relapse rates[44]. If a second voiding diary indicates that nocturnal urinary production is not reduced, consider a dose increase. As a rule of thumb, one third of unselected enuretic children are reliably dry as long as they take the drug, one third has a partial response and one third is not helped at all. Fluid overload (water intoxication) is potentially the most serious complication with desmopressin. It is associated with overdrinking at bedtime and its symptoms include headache, nausea, hyponatraemia, cerebral oedema, and convulsions. Overall desmopressin has an excellent safety profile with very few significant adverse events reported[45]. The reported success rates of DDAVP treatment for enuresis have ranged from 10% to 65%, but as many as 80% of patients relapse after treatment[46]. Depression of endogenous ADH secretion is not a concern as children who have used DDAVP for as long as 1 year have demonstrated the ability to concentrate their urine appropriately in response to a water deprivation challenge. It seems reasonable at least to consider a trial of withdrawal of DDAVP at 3- to 6-mo intervals.

In comparison to arousal alarms, treatment effects were not sustained after discontinuation of therapy (the rate of failure or relapse was 65% and 46% with desmopressin and alarms, respectively; relative risk of failure 1.42, 95%CI: 1.05-1.91). Comparison with some tricyclic drugs (*e.g.*, amitriptyline) suggests that they might be as effective as desmopressin although in two trials, children were less likely to achieve 14 dry nights with imipramine than desmopressin (RR 0.44, 95%CI: 0.27-0.73) but there was not enough information about subsequent relapse[47]. There were more side effects with the tricyclics.

The British National Formulary currently suggests that drug therapy is not usually appropriate for children under 7 years of age and should be reserved for children in whom alternative measures have failed.

### *Failure to therapy*

Inability to achieve > 50% improvement in symptoms is defined as resistant to therapy. If this happens despite an adequate trial of treatment with an enuresis alarm (*i.e.*, three months) and/or desmopressin (at a dose of 0.4 mg) and in absence of any concern regarding the family/child's motivation then referral to a healthcare professional who specializes in the management of bedwetting (*e.g.*, developmental behavioral pediatrician, pediatric urologist) may be warranted.

Possible reasons for lack of response include: (1) Overactive bladder; (2) Underlying disease (*e.g.*, diabetes mellitus/diabetes insipidus); (3) Occult constipation; (4) Sleep apnea; (4) Incorrect use of alarm; and (5) Social and emotional factors.

If on additional evaluation (which may include repeat bladder diary, ultrasound scan (if not done before), rectal examination/abdominal X ray for constipation) no underlying aetiology is found then combination therapy or switch to imipramime, may be considered. For children with suspected day and night detrusor over activity/small functional bladder capacity, a combination of oxybutynin and desmopressin may be indicated (level 2, grade B).

Imipramine (a tri-cyclic anti-depressant) in a single bedtime dose of 1.0-2.5 mg/kg had been used for many years as a third line agent for enuresis. Tricyclic antidepressants (TCAs, *e.g.*, imipramine, amitriptyline and desipramine) decrease the amount of time spent in REM sleep, stimulate vasopressin secretion, and relax the detrusor muscle although the exact mechanism of action in treating enuresis is unknown. Major rare significant adverse effects include cardiotoxicity and hepatotoxicity. Minor side-effects are related to their anti-cholinergic actions and include postural hypotension, dry mouth, constipation, perspiration, tachycardia, nausea, lethargy and insomnia. A pretreatment electrocardiogram may be obtained to detect any underlying rhythm disorder. Treatment can be continued for 4-6 mo. In a systematic review, compared with placebo, treatment with TCA was associated with reduction of approximately one wet night per week[48].

### *Other drugs*

Anti-cholinergic drug tolterodine, oxybutynin and propiverine is in fact useful as an add-on therapy in enuretic children who have not responded to desmopressin alone[49]. They carry a risk for constipation and for UTI due to the accumulation of residual urine. Other drugs, including indomethacin, phenmetrazine, amphetamine sulfate, ephedrine, atropine, furosemide, diclofenac, and chlorprothixene have been tried in the treatment of nocturnal enuresis. A 2012 systematic review of randomized trials of drugs other than tricyclic antidepressants and desmopressin found that although indomethacin, diclofenac, and diazepam were better than placebo in reducing the number of wet nights, none of the drugs was better than desmopressin[50]. Atomoxetine used in ADHD has been

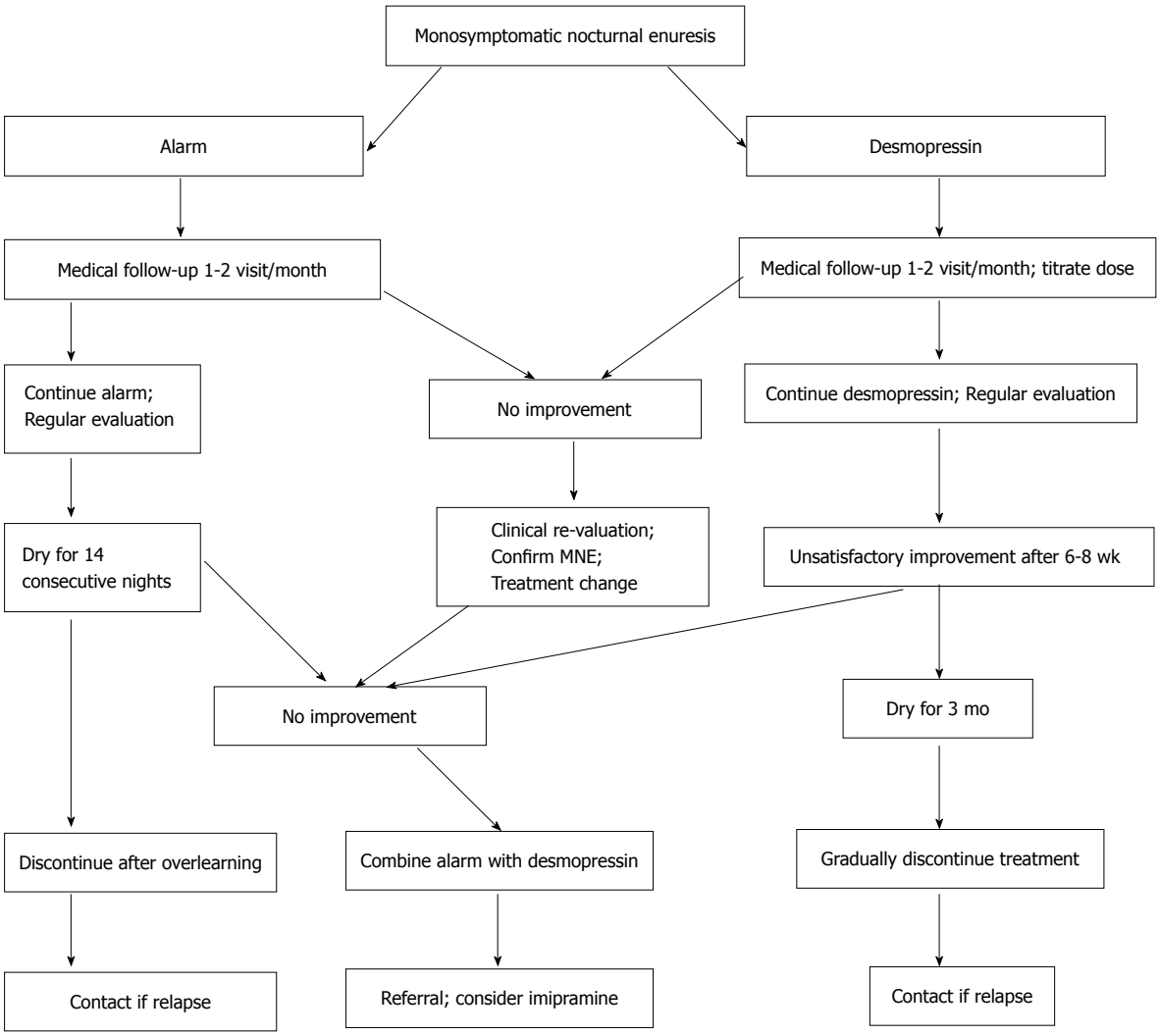


**Figure 3 Treatment flowchart for monosymptomatic nocturnal enuresis.** MNE: Monosymptomatic enuresis.

found to decrease frequency of bedwetting among children with enuresis with or without[51].

**In summary**: Differentiating between MNE and NMNE forms the cornerstone in the management of these children. Once MNE is identified and initial steps like counseling, bladder diarys, *etc.*, have failed the first treatment for the family who is well-motivated and well informed is the enuresis alarm. Desmopressin is the first line treatment for families who are not sufficiently motivated to use the alarm, who have recently used the alarm (correctly) without success or who are considered unlikely to comply with alarm treatment. Anti-cholinergic therapy has the greatest chance of success in the child with signs of detrusor over activity, *i.e.*, low daytime voided volumes. If desmopressin, the alarm and the anti-cholinergic treatment have all been tried without success, or have been judged unsuitable, the cautious use of imipramine may be considered (Figure 3).

**Follow-up:** Following successful treatment with either the alarm or desmopressin, patients should be advised to contact the clinic if relapse is experienced after discontinuation of therapy. If relapse occurs, desmopressin, alarm, or combined therapy should be re-considered. The most likely fundamental reason for not responding to alarm or desmopressin therapy is that the actual diagnosis is NMNE and not MNE. When a detailed history is obtained, the majority of these children have at least subtle daytime symptoms. If a patient is treatment-resistant and a bladder diary has not been completed, it is imperative this is undertaken or to refer the child to a specialty center as OAB and dysfunctional voiding may be present.

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ORIGINAL ARTICLE

Basic Study

# Constitutive renal Rel/nuclear factor-κB expression in Lewis polycystic kidney disease rats

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Author contributions: Schwensen KG conducted the animal study, performed sample collection and histological staining; Ta MHT performed the immunofluorescence, Western blotting, and qPCR experiments, analyzed data and drafted the manuscript; Huso DL and Watnick T developed the Pkd2 knockout mouse model and provided paraffin embedded sections that were utilized for staining; Liuwantara D assisted with data interpretation and provided technical guidance with experimental methods; Rangan GK conceived of the study, conducted the animal study, performed sample collection and histological staining and reviewed and edited the manuscript; all authors read and approved of the final manuscript.

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## Abstract

**AIM:** To determine the temporal expression and pattern of Rel/nuclear factor (NF)-κB proteins in renal tissue in polycystic kidney disease (PKD).

**METHODS:** The renal expression of Rel/NF-κB proteins was determined by immunohistochemistry, immunofluorescence and immunoblot analysis in Lewis polycystic kidney rats

(LPK, a genetic ortholog of human nephronopthsis-9) from postnatal weeks 3 to 20. At each timepoint, renal disease progression and the mRNA expression of NF-κB-dependent genes (TNFα and CCL2) were determined. NF-κB was also histologically assessed in human PKD tissue.

**RESULTS:** Progressive kidney enlargement in LPK rats was accompanied by increased renal cell proliferation and interstitial monocyte accumulation (peaking at weeks 3 and 10 respectively), and progressive interstitial fibrosis (with α smooth muscle actin and Sirius Red deposition significantly increased compared to Lewis kidneys from weeks 3 to 6 onwards). Rel/NF-κB proteins (phosphorylated-p105, p65, p50, c-Rel and RelB) were expressed in cystic epithelial cells (CECs) of LPK kidneys as early as postnatal week 3 and sustained until late-stage disease at week 20. From weeks 10 to 20, nuclear p65, p50, RelB and cytoplasmic IκBα protein levels, and TNFα and CCL2 expression, were upregulated in LPK compared to Lewis kidneys. NF-κB proteins were consistently expressed in CECs of human PKD. The DNA damage marker γ-H2AX was also identified in the CECs of LPK and human polycystic kidneys.

**CONCLUSION:** Several NF-κB proteins are consistently expressed in CECs in human and experimental PKD. These data suggest that the upregulation of both the canonical and non-canonical pathways of NF-κB signaling may be a constitutive and early pathological feature of cystic renal diseases.

**Key words:** Inflammation; Nuclear factor-κB; Polycystic kidney disease; Tumour necrosis factor alpha; Chemokine CCL2



**Core tip:** Until now, there has been limited information regarding the specific nuclear factor (NF)-κB proteins involved in polycystic kidney disease (PKD) and their expression throughout disease progression. Our study demonstrated that a diverse array of NF-κB proteins is expressed in the renal cyst-lining cells of a chronic rodent model of PKD, and that NF-κB expression is constitutive over time. NF-κB was also identified in human PKD, suggesting that NF-κB upregulation is common to renal cystic disease models. Our data suggest that components of both the canonical and non-canonical NF-κB pathway are upregulated in PKD. Future studies should be directed at verifying whether specific NF-κB inhibition can attenuate interstitial inflammation and cyst growth, and slow the decline in renal function in *in vivo* models of PKD.

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## INTRODUCTION

Polycystic kidney diseases (PKD) are a group of genetic disorders characterized by the formation of multiple renal cysts and an increased life-time risk for kidney failure[1]. The two most common forms, autosomal dominant and recessive PKD (ADPKD and ARPKD), are due to mutations in *PKD1/2* and *PKHD1* respectively[2-4]. These genes encode proteins that are localized to the cilia of most cells within the body, including renal tubule epithelia[5,6]. Interstitial inflammation is a universal histological feature associated with renal cyst growth and formation[7-9], and is possibly mediated by the release of pro-inflammatory cytokines from cyst-lining epithelial cells (CECs)[8]. Abnormalities in apoptosis and increased proliferation of CECs[7,10], interstitial fibrosis[11] and hypertension[12] are also typical features of PKD.

The nuclear factor (NF)-κB system is a key regulator of pro-inflammatory and pro-apoptotic gene transcription[13,14]. The Rel proteins, namely p65 (RelA), RelB and c-rel, contain transcription-binding domains (TADs) that allow them to bind DNA[13,14]. In contrast, p50 (NF-κB1) and p52 (NF-κB2), which are derived from the breakdown of p105 and p100 respectively, do not possess TADs and therefore must bind to Rel proteins in order to modulate transcription[13,15]. In the inactive state, NF-κB proteins normally exist as dimers, and are bound to inhibitor of κB (IκB) proteins in the cytoplasm[13]. Upon activation by certain stimuli, IκB kinase (IKK) proteins are activated, phosphorylating the IκB proteins and leading to their degradation, thus freeing the NF-κB proteins to translocate to the nucleus and regulate transcription[13,14]. NF-κB signaling can be broadly classified as canonical or non-canonical[13,16]. The canonical pathway is activated by a wide range of stimuli including tumor necrosis factor (TNF)α and lipopolysaccharide[13,17], and typically involves the p65:p50 dimer and IκBα[13,16,18]. The non-canonical pathway is known to be stimulated by fewer stimuli (*e.g.*, CD40 ligand and lymphotoxin-β2[16]) and usually implicates the RelB:p52 dimer in mediating lymphoid organ development[13,16].

There is an overlap between NF-κB-regulated genes and the pathophysiological features of PKD, such as inflammation (*e.g.*, *TNFα, CCL2*), cell growth (*e.g.*, *cyclin D1*), apoptosis (*e.g.*, *Bcl-xL*) and hypertension (*e.g.*, *ANGII*)[8,9,12,17,19]. These commonalities provide a theoretical basis for the involvement of NF-κB in PKD. Recent studies have provided preliminary evidence for NF-κB involvement in the pathogenesis of cystic renal disease. For example, the use of small interfering RNA (siRNA) to over-express or delete ciliary proteins *in vitro*, leads to upregulation of NF-κB signaling[20,21]. Park *et al*[22] demonstrated that *PKD2* transgenic mice have higher levels of renal NF-κB proteins and phosphorylated-IKKα/β compared to wild type controls. Moreover, upregulated NF-κB expression has been identified in the CEC nuclei in *Pkd1*$^{-/-}$ and *PKD2* transgenic mice[22], and in human ADPKD[22].

Despite the complexity of the NF-κB system, the previous studies of NF-κB in PKD mainly or solely focused on p65, and in some cases did not specify the

particular NF-κB subunits that were investigated[22,23]. Therefore, the first aim of the current study was to examine the expression and localization of a spectrum of NF-κB proteins, in a chronic disease model of PKD and in human cystic renal disease. We investigated the expression of p65, p50, RelB and c-rel, as well as IκBα and phosphorylated p105 (P-p105, which is synthesized prior to p50 production and accordingly is a marker of NF-κB upregulation[24]). In addition, cyst development in ADPKD is progressive, beginning *in utero* and continuing through to the later decades of life[9], but no studies have examined whether the NF-κB system changes throughout disease progression in PKD. Thus, the second aim of this study was to characterize NF-κB expression over the time-course of disease in the Lewis polycystic kidney (LPK) rat (a chronic model of ARPKD[25]). Based on data showing that the expression of a NF-κB-dependent gene, *TNFα,* is upregulated in *cpk* mice and increases further over time[26], and previous correlations between TNFα and NF-κB signaling in *in vitro* models of PKD[27], we hypothesized that NF-κB protein expression is elevated in LPK kidneys compared to control kidneys in the early stages of PKD, and increases incrementally throughout the course of the disease.

## MATERIALS AND METHODS

### *Lewis polycystic kidney disease model of PKD*

The LPK rat is a genetic ortholog of *NPHP9* (human nephronophthisis) due to a point mutation in *Nek8*[28]. It is characterized by post-natal distal nephron and collecting duct ectasia which progress over approximately 20 wk, and is associated with hypertension and renal failure[25]. In this study, LPK rats and Lewis/SSN rats were obtained from the breeding colony at Westmead Hospital. This colony was established in 2008 from a single founder homozygous breeding pair from the Animal Resources Centre (Perth, Western Australia). The colony was maintained by mating homozygous male and female breeder pairs. Animals were housed under standard conditions and allowed food and water ad libitum at the Animal Care Department at Westmead Hospital. All protocols and procedures were approved by the Western Sydney Local Health District Animal Ethics Committee, (Protocol number 4100). Since disease is more severe in male than in female LPK rats[25], for this study, male LPK rats were sacrificed at postnatal weeks 1, 2, 3, 4, 6, 10, 16 and 20 ($n$ = 6-9 per timepoint) and were compared to male Lewis animals at the same timepoints ($n$ = 3-6 per timepoint).

### *Assessment of kidney enlargement and renal function in LPK rats*

Kidney enlargement was determined using the kidney to body weight ratio (KW:BW) at the time of tissue collection. Renal function was assessed in blood at the time of sacrifice, and was analyzed by the Institute of Clinical Pathology and Medical Research. To assess proteinuria and creatinine clearance (CrCl), rats were placed in metabolic cages for 16 h. Creatinine clearance was calculated using CrCl = [Urine creatinine (μmol/L) × Urine volume (mL/min)]/Serum creatinine (μmol/L), and was corrected for body weight. Rats were euthanized as previously described[29].

### *Immunohistochemistry*

Coronal kidney slices were fixed in either 37 g/L formaldehyde or methyl Carnoy's solution for 24 h, then paraffin embedded. For immunohistochemistry, 4 μm sections were deparaffinized, blocked with 0.03 g/mL hydrogen peroxide, and antigen retrieval was performed by microwave oven heating (for formalin slides only, 100% power, 10 min in 1 × Antigen Decloaker, Biocare Medical, CA). Sections were blocked with 100 mL/L goat serum, and incubated with primary antibodies for 1 h overnight at 4 ℃. The primary antibodies used were: (1) anti-Ki67 to assess proliferation (1:100, ab16667, Abcam, Cambridge, United Kingdom); (2) anti-ED-1 for $CD68^+$ monocytes to assess inflammation (1:400, MCA341R, Serotec, United Kingdom); (3) anti-α smooth muscle actin (α-SMA) to assess myofibroblast accumulation (and also a marker of vascular smooth muscle cells, 1:4000, A2547, Sigma-Aldrich, St. Louis, MO); and (4) anti-phosphorylated-p105 (P-p105) to assess NF-κB expression (1:50, #4808 Cell Signaling Technology, Danvers, MA). Secondary biotinylated antibodies were applied for 30 min at room temperature (anti-mouse, 1:200, 65-6440; anti-rabbit, 1:200, 65-6140, Life Technologies, Carlsbad, CA). Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) was applied for 20 min, followed by diaminobenzidine. Sections were counterstained with methyl green (Sigma-Aldrich) then dehydrated. To assess interstitial fibrosis, Sirius Red staining was performed on methyl Carnoy's fixed sections with 1 g/L Direct Red 80 and 1 g/L Fast Green FCF (Sigma-Aldrich) for 24 h. To quantify immunohistology, whole slide digital images (20 × magnification) were acquired using a scanner (ScanScope CS2, Aperio, CA). Percentage cyst volume was assessed in Periodic Acid Schiff (PAS) stained sections by whole-slide digital analysis in Aperio ImageScope (v11.2.0.780).

### *Immunofluorescence*

For immunofluorescence, formalin-fixed slides were deparaffinized and antigen retrieval was performed by microwave oven heating as described above. Sections were soaked in Tris Buffered Saline (TBS), 4 mL/L TritonX, for 30 min then blocked with 30 g/L BSA in TBS, 2 g/L Tween20 for 1 h. Slides were incubated with primary antibodies for 1h overnight at 4 ℃: (1) p65 (1:100, #8242, Cell Signaling); (2) p50/p105 (1:100, ab7971, Abcam); (3) RelB (1:100, bs-3562R, Bioss Antibodies, Woburn, MA); (4) c-rel (1:100, orb5913, Biorbyt, Cambridge, United Kingdom); and (5) γ-H2AX (1:100, ab2893, Abcam). Secondary antibody (1:200, Alexa Fluor 546 goat anti-rabbit IgG, A-11010, Life Technologies) was applied for 30 min at room temperature, following by DAPI for 5 min. Slides were mounted using Fluorescence Mounting Medium (Dako, Glostrup, Den-

mark). Immunofluorescence was assessed using an Olympus BX53/DP80 microscope (Olympus Corporation, Shinjuku, Japan) and images were taken at 20 × and 40 × magnification using the software (cellSens, v1.6, Olympus).

### *Western blot*

Nuclear and cytosolic extracts were obtained from 100 mg of kidney cortex from Lewis and LPK animals at weeks 3, 10 and 20, using a previously described method[30] and stored at -80 ℃. Protein concentration of the extracts was assessed using the DC Protein Assay (Bio-Rad). Western blot was performed as previously described[31]. Primary antibodies used were: p50/105 (1:1000, ab7971, Abcam), p65 (1:1000, #8242, Cell Signaling Technology, Danvers, MA), RelB (1:1000, #4922, Cell Signaling), IκBα (1:1000, #4814, Cell Signaling), β-actin (1:1000, #4970, Cell Signaling), and GAPDH (1:1000, #5174, Cell Signaling). Densitometry was quantified using ImageJ (v1.47, National Institutes of Health, United States) and normalized using β-actin (for nuclear extracts) or GAPDH (for cytoplasmic extracts).

### *Quantitative real-time polymerase chain reaction*

Quantitative real-time polymerase chain reaction (qPCR) was performed to assess the mRNA expression of two pro-inflammatory genes, *TNFα* and chemokine (C-C motif) ligand 2 *(CCL2)*. Renal tissue was snap-frozen in liquid nitrogen and stored at -80 ℃. RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands). RNA was reverse-transcribed into cDNA (SuperScript Ⅲ First-Strand Synthesis System, Thermo Fisher Scientific, Waltham, MA) using oligo(dT) and dNTP, and using the cDNA Synthesis Mastermix according to manufacturer's instructions. Real-time quantitative PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher) on a Bio-Rad CFX96 machine (Bio-Rad Laboratories, Hercules, CA). The PCR primers were: TNFα (forward: 5′ GTC GTA GCA AAC CAC CAA GC 3′, reverse: 5′ TGT GGG TGA GGA GCA CAT AG 3′)[32], CCL2 (forward: 5′ AGC CCA GAA ACC AGC CAA CTC 3′, reverse: 5′ GCC GAC TCA TTG GGA TCA TCT T 3′)[33], and GAPDH (forward: 5′ GAA CAT CAT CCC TGC ATC CA 3′, reverse: 5′ CCA GTG AGC TTC CCG TTC A 3′)[34]. PCR parameters were 95 ℃ for 2 min and 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 30 s for 40 cycles; melting temperature was measured between 65 ℃ and 95 ℃. Data were analyzed using CFX Manager software (v3.1.1517.0823, 2012 release, Bio-Rad). Gene expression was quantified using the ΔΔCT method. The mRNA quantities of *TNFα* and *CCL2* in each sample were normalized using *GAPDH* mRNA quantity.

### *Assessment of NF-κB expression in human PKD*

Renal tissue was obtained from two ADPKD patients (ID no. P17 and P18) and one ARPKD patient (ID no. ARPKD1). Kidney tissue was also obtained from two non-PKD patients (ID no. C1 and C2; both kidneys were removed due to renal cancer, and the non-cancerous portions used as controls). All patients provided written informed consent, and the study was approved by the Human Research Ethics Committee at Westmead Hospital (HREC/09/WMEAD/305; SSA/12/WMEAD/327). Samples were paraffin-embedded, and PAS staining, immunohistochemistry and immunofluorescence were performed as described for LPK kidneys.

### *Statistical analysis*

Results were presented as mean ± SD. The data were analyzed with the JMP statistical software package (v4.04, SAS institute, Carey, NC, United States) and graphed in GraphPad Prism (v6.04 for Windows, GraphPad Software, San Diego, CA). Comparisons between the experimental groups were performed by ANOVA, followed by a post-hoc analysis with the Tukey-Kramer HSD test. A *P*-value of $< 0.05$ was interpreted as statistically significant.

## RESULTS

### *Time-course of renal disease progression in LPK rats*

**Body weight and kidney enlargement:** Body weight increased steadily in both experimental groups from weeks 1 to 20, but was lower in LPK animals (Figure 1A). In LPK rats, the KW:BW was increased compared to Lewis rats as early as postnatal week 1 and continued to rise until week 16 (Figure 1B). The rate of increase in KW:BW was greatest between weeks 6 and 10 (239% increase) whereas between weeks 10 and 20 only a further 16% increase was observed (Figure 1B).

**Histological pattern of renal disease:** Qualitative histological analysis showed that there was progressive dilatation of collecting ducts and distal tubules over time in LPK kidneys (Figure 2). At week 1, cystic renal disease was mild, characterized by focal areas of rounded collecting duct dilatation in the corticomedullary region. From week 2 onwards, the rounded dilatation developed into rectangular elongation. As shown in Table 1, the mean dimensions (width and length) of the cyst tubular dilatation increased progressively from week 1 to 20. These histological changes were accompanied by time-dependent increases in renal interstitial inflammation (ED-1 immunohistochemistry) and fibrosis (α-SMA immunohistochemistry and Sirius Red staining) in LPK rats (Table 1). As previously shown[35], cell proliferation (Ki-67 positive cells) peaked at week 3 in LPK rats. Interstitial ED-1 and α-SMA were highest at week 10 (Table 1). Collagen deposition (as measured by Sirius Red) was elevated in LPK kidneys from week 6 onwards (Table 1).

**Renal function:** LPK rats developed an increase in 24 h urine volume compared to Lewis rats from week 10 (Table 2). A decline in renal function was indicated by the elevated levels of serum creatinine in LPK compared to Lewis rats from week 10 onwards (Table 2). Creatinine clearance was decreased in LPK rats compared to Lewis at weeks 10 and 16. Serum urea was elevated in LPK rats at all timepoints (Table 2).

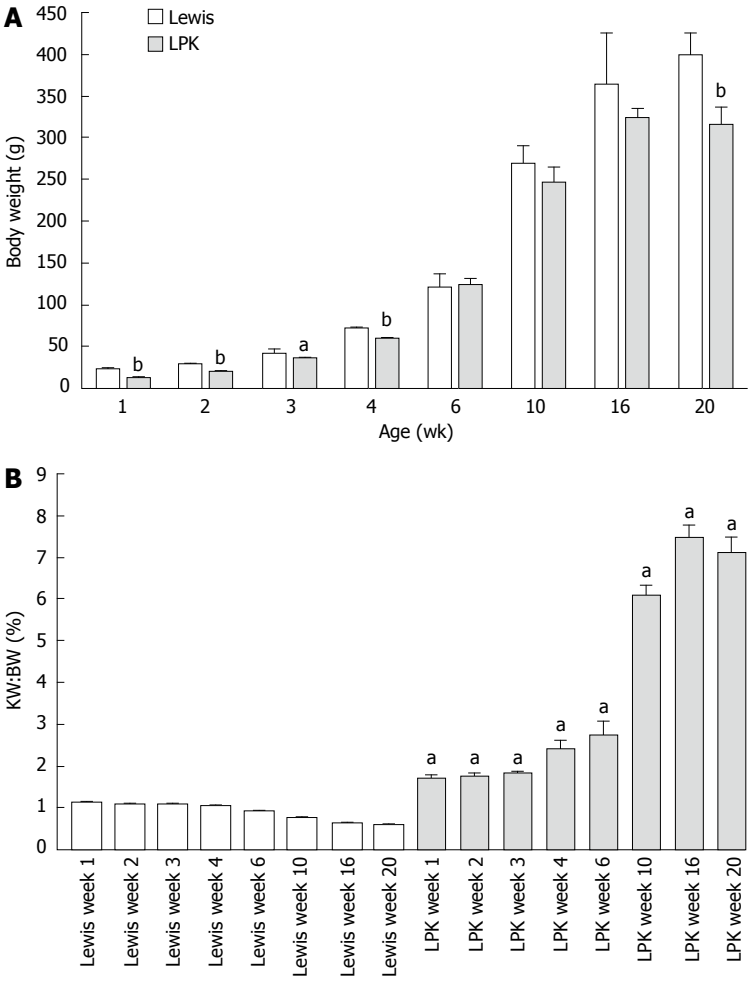



**Figure 1 Body weight and kidney weight over time in Lewis and Lewis polycystic kidney rats.** A: Body weight of male Lewis and LPK rats from weeks 1 to 20. Data as mean ± SD. $^{a}P < 0.05$ *vs* age-matched Lewis animals; $^{b}P < 0.01$ *vs* age-matched Lewis animals; B: Percentage two kidney weight to body weight ratio (KW:BW) of male Lewis and LPK rats from weeks 1 to 20. Data as mean ± SD. $^{a}P < 0.05$ *vs* age-matched Lewis animals. LPK: Lewis polycystic kidney.

**Table 1 Histological analysis of Lewis and Lewis polycystic kidney rats from weeks 1 to 20**

| Parameter | | Week 1 | Week 3 | Week 6 | Week 10 | Week 16 | Week 20 |
|---|---|---|---|---|---|---|---|
| Cyst length (μm) | Lewis | ND | ND | ND | ND | ND | ND |
| | LPK | 106 ± 4 | 347 ± 88 | 938 ± 280 | 1548 ± 237 | 2252 ± 436 | 3718 ± 922 |
| Cyst diameter (μm) | Lewis | ND | ND | ND | ND | ND | ND |
| | LPK | 69 ± 18 | 101 ± 19 | 279 ± 71 | 442 ± 81 | 681 ± 121 | 1119 ± 256 |
| Ki-67 (%) | Lewis | 24.3 + 8.3 | 1.9 + 0.6 | 2.4 + 1.5 | 0.2 + 0.1 | 0.3 + 0.1 | 0.2 ± 0.02 |
| | LPK | 7.0 ± 3.8[a] | 9.9 + 2.6[a] | 2.2 ± 1.5 | 1.1 ± 0.4[a] | 1.9 ± 0.8[a] | 0.8 ± 0.3[a] |
| ED-1 (%) | Lewis | 1.87 ± 0.61 | 1.26 ± 0.20 | 1.32 ± 0.95 | 0.56 ± 0.08 | 0.22 ± 0.11 | 0.16 ± 0.02 |
| | LPK | 1.58 ± 0.15 | 3.81 ± 1.02[a] | 4.87 ± 1.21[a] | 12.37 ± 4.10[a] | 0.81 ± 0.38[a] | 0.84 ± 0.24[a] |
| Sirius Red (%) | Lewis | 0.86 ± 0.38 | 4.32 ± 1.58 | 1.67 ± 0.52 | 2.91 ± 0.77 | 0.51 ± 0.43 | 0.64 ± 0.40 |
| | LPK | 1.96 ± 1.15 | 2.88 ± 1.51[a] | 5.92 ± 4.20[b] | 16.63 ± 8.12[b] | 10.34 ± 5.82[b] | 18.24 ± 6.00[b] |
| α-SMA (%) | Lewis | 17.2 ± 2.12 | 1.68 ± 0.42 | 1.87 ± 0.32 | 0.98 ± 0.86 | 1.04 ± 0.06 | 1.09 ± 0.13 |
| | LPK | 22.4 ± 2.80 | 8.70 ± 3.70[a] | 8.98 ± 3.41[a] | 20.18 ± 3.54[a] | 13.75 ± 0.99[a] | 13.28 ± 1.68[a] |

Data expressed as mean ± SD; $^{a}P < 0.05$ *vs* age-matched Lewis male animals; $^{b}P < 0.01$ *vs* age-matched Lewis male animals. α-SMA: Alpha-smooth muscle actin; BrdU: 5-Bromo-2'-deoxyuridine; ND: Not determined.

### *Time-course of renal Rel/NF-κB protein localization in LPK rats*

**p50:** In kidneys from Lewis rats, p50 expression was weak and diffuse and localized predominantly to the cytoplasm of tubular cells (Figure 3). In LPK kidneys, this pattern of expression was maintained, but in addition there was strong staining of epithelial cells lining the dilated tubular cystic segments (Figure 3). The staining, which occurred in the majority of cysts, was detected as early as week 3 and remained persistent at weeks 10 and 20. Populations of intensely staining interstitial cells were also noted in LPK kidneys in the outer medulla at weeks 6 and 20 (data not shown).

**P-p105:** In Lewis rats, at week 3, P-p105 staining was observed in focal areas of tubules and in selected cortical glomerular cells (Figure 4). This pattern of staining was stronger at weeks 10 and 20. In LPK rats,

**Table 2 Renal function for male Lewis and Lewis polycystic kidney rats from weeks 3 to 20**

| Parameter | | Week 3 | Week 6 | Week 10 | Week 16 | Week 20 |
|---|---|---|---|---|---|---|
| 24 h urine volume (mL) | Lewis | ND | 6 ± 1 | 9 ± 2 | 9 ± 2 | ND |
| | LPK | ND | 7 ± 1 | 25 ± 2[b] | 23 ± 9[b] | ND |
| Serum creatinine (μmol/L) | Lewis | 24 ± 3 | 22 ± 5 | 29 ± 10 | 24 ± 1 | 27 ± 5 |
| | LPK | 20 ± 2[a] | 28 ± 14 | 33 ± 3[b] | 63 ± 9 | 191 ± 38[b] |
| Endogenous CrCl (mL/min) | Lewis | ND | 0.5 ± 0.2 | 1.8 ± 0.3 | 2.5 ± 0.6 | ND |
| | LPK | ND | 0.5 ± 0.2 | 0.8 ± 0.1[b] | 0.6 ± 0.2[b] | ND |
| CrCl/BW (fold-change over Lewis) | Lewis | ND | 1 ± 0.4 | 1.6 ± 0.1 | 1.7 ± 0.3 | ND |
| | LPK | ND | 0.9 ± 0.3 | 0.8 ± 0.1[b] | 0.4 ± 0.1[b] | ND |
| Serum urea (mmol/L) | Lewis | 5 ± 1 | 6 ± 1 | 6 ± 1 | 6 ± 1 | 8 ± 1 |
| | LPK | 7 ± 0[b] | 8 ± 1[b] | 14 ± 1[b] | 22 ± 3[b] | 44 ± 4[b] |

Data expressed as mean ± SD; [a]$P < 0.05$ *vs* age-matched Lewis male animals; [b]$P < 0.01$ *vs* age-matched Lewis male animals. BW: Body weight; CrCl: Creatinine clearance; ND: Not determined.

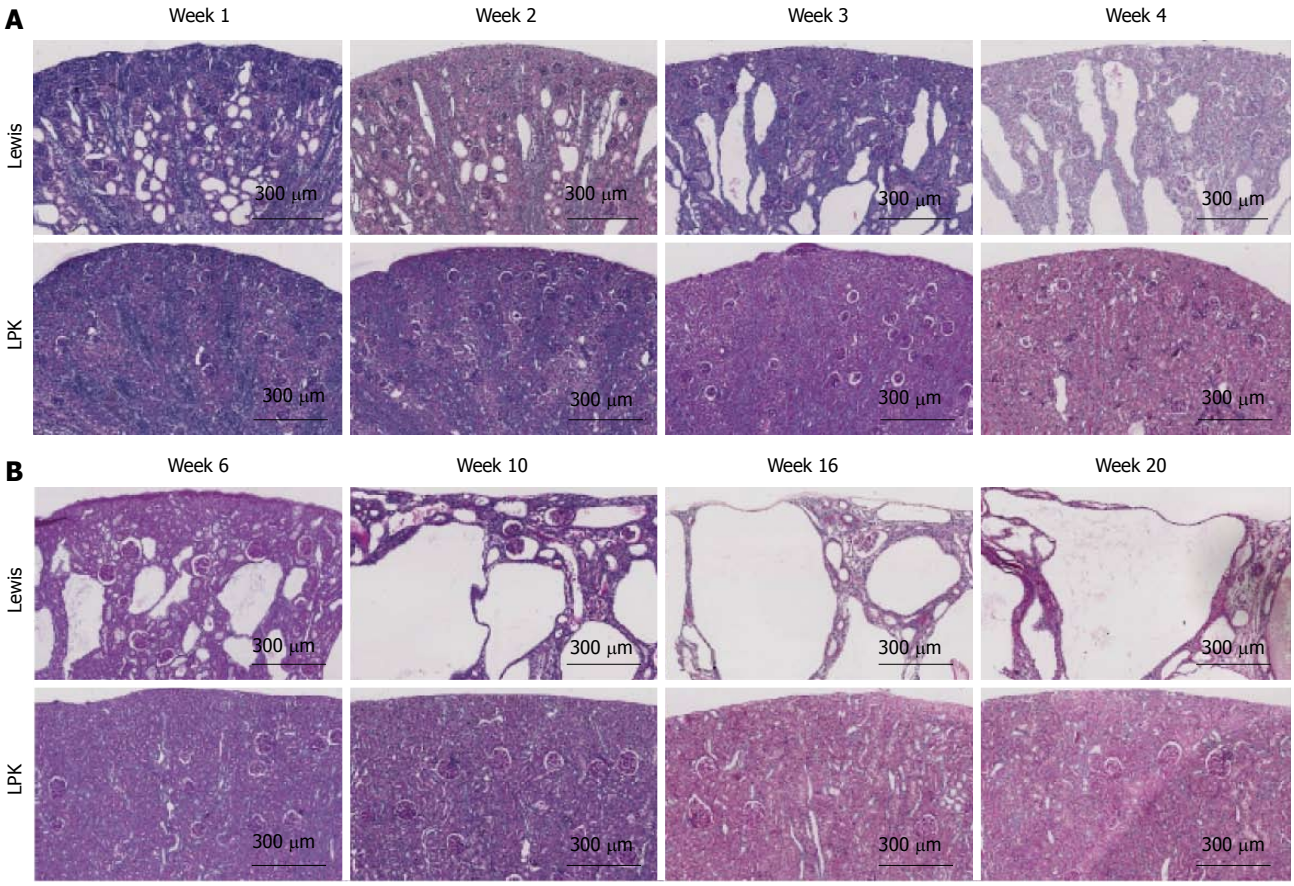



**Figure 2 Whole-slide digital images of Periodic Acid Schiff-stained sections of Lewis control and Lewis polycystic kidney rat kidneys, at (A) weeks 1 to 4, and (B) weeks 6 to 20.** LPK: Lewis polycystic kidney.

P-p105 was strongly expressed in cystic epithelial cells at week 3, 10 and 20 in the majority of cysts (Figure 4).

**p65:** In Lewis rats, p65 was localised to the cytoplasm of tubules. Expression was strongly upregulated at week 3, but declined at weeks 10 and 20 (Figure 5). In LPK kidneys, p65 was strongly expressed in the cytoplasm of cortical CECs in the majority of cysts at all timepoints, starting from week 3 (Figure 5).

**RelB:** In Lewis rats, RelB expression in the kidney was weak at all timepoints (Figure 6). In contrast, in LPK there was strong staining of cysts at mid to late disease, particularly at weeks 10 and 16 (Figure 6).

**c-rel:** In Lewis rats, the renal expression of c-rel was localized to the nuclei and cytoplasm of tubules and interstitial cells (Figure 7). In control animals, nuclear c-rel expression was higher at week 3, declining at weeks 10 and 20, but cytoplasmic c-rel expression remained consistent over time. In LPK rats, cystic epithelial cell staining was evident at weeks 3 to 20, in the nuclei and

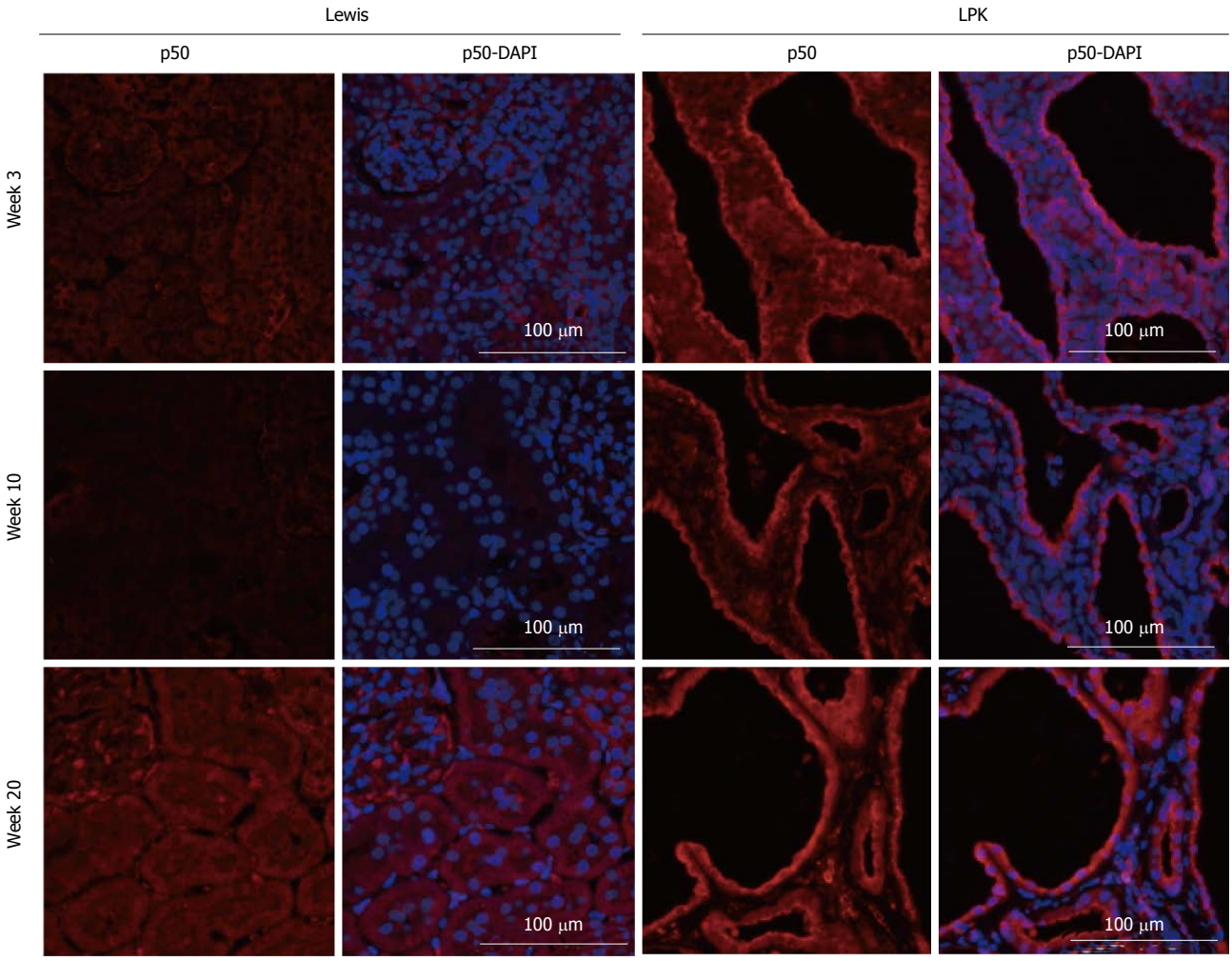



**Figure 3 Immunofluorescence staining for p50 (red) at weeks 3, 10 and 20 in Lewis and Lewis polycystic kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue). LPK: Lewis polycystic kidney.

cytoplasm (Figure 7).

### *Time-course of nuclear Rel/NF-κB and cytoplasmic IκBα expression in the kidney*

Western blotting of nuclear kidney extracts found that p65 expression was increased in LPK rats, peaking at week 10, when it was 18-fold higher than in Lewis animals (Figure 8). Similarly, p50 was elevated in LPK rats compared to the Lewis group, peaking at week 20 (Figure 8). For RelB, at week 3, nuclear expression was similar between Lewis and LPK rats (Figure 9). However, at weeks 10 and 20, there was a marked upregulation in nuclear RelB expression in LPK rats. Cytoplasmic IκBα displayed the same temporal pattern of expression as nuclear RelB in LPK kidneys (Figure 9).

### *Time-course of renal NF-κB-dependent gene expression in LPK rats*

The upregulation of Rel/NF-κB proteins in LPK rats was accompanied by time-dependent increases in the mRNA expression of NF-κB-dependent genes (*CCL2* and *TNFα* mRNA), which were elevated in LPK rats compared to the Lewis group, particularly at the late stages of disease (Figure 10).

### *Renal expression of NF-κB in human ADPKD and ARPKD*

Human ADPKD and ARPKD kidneys displayed cystic dilatations of varying sizes, lined by either flattened single-layer, or hyperplastic epithelial cells (Figure 11). Abnormal glomeruli, interstitial fibrosis and large numbers of infiltrating cells were also observed. By immunofluorescence, in control kidneys, p50 staining was intensely expressed in proximal tubule brush borders, but weak in the glomeruli and in renal tubule cytoplasm (Figure 12). In ADPKD tissue, p50 staining was strong in the cytoplasm and moderate in the nuclei of CECs. Strong expression was also detected in intraluminal and interstitial cells. A similar pattern of staining was observed in ARPKD, with strong expression in CEC cytoplasm and moderate expression in nuclei (Figure 12). Among the CECs, p50 expression was notably more intense in areas of hyperplasia than in areas where the epithelial layer was single-cell thick. Staining was also strong and diffuse in interstitial cells (Figure 12). A similar pattern of staining was observed for P-p105, p65 and RelB (Figures 11 and

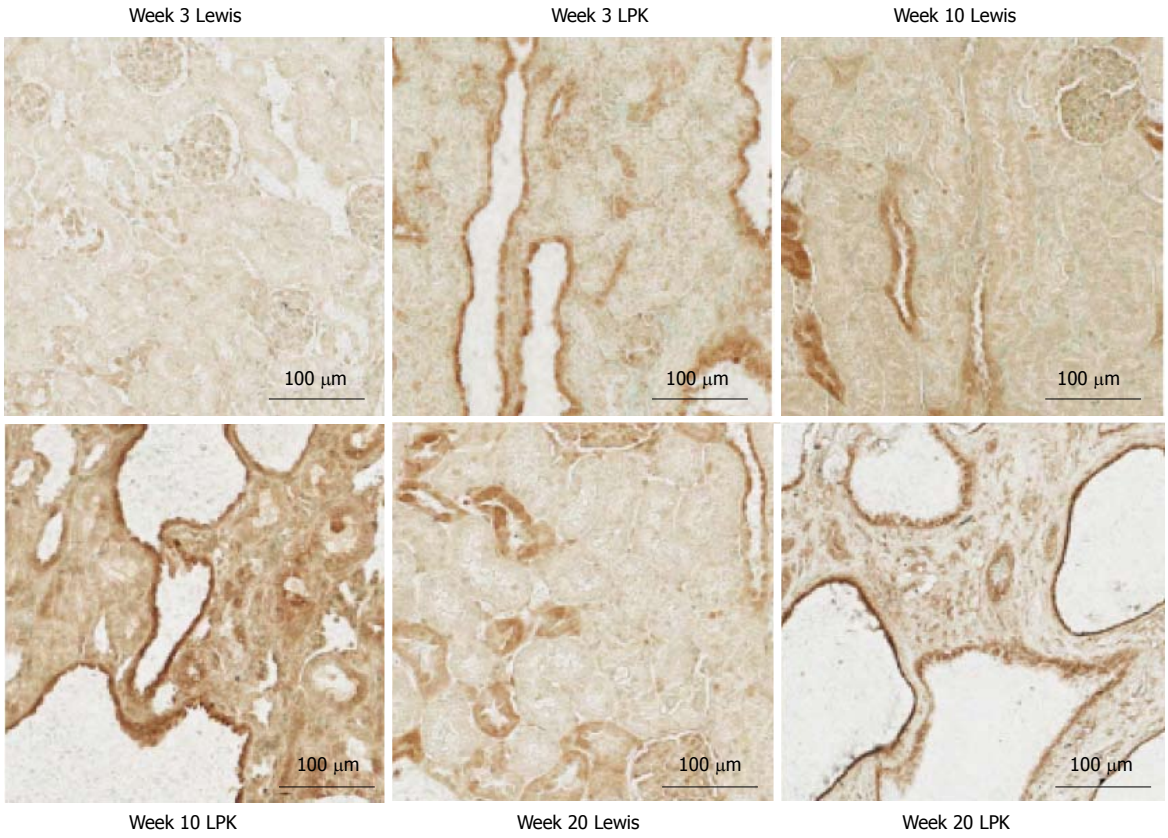
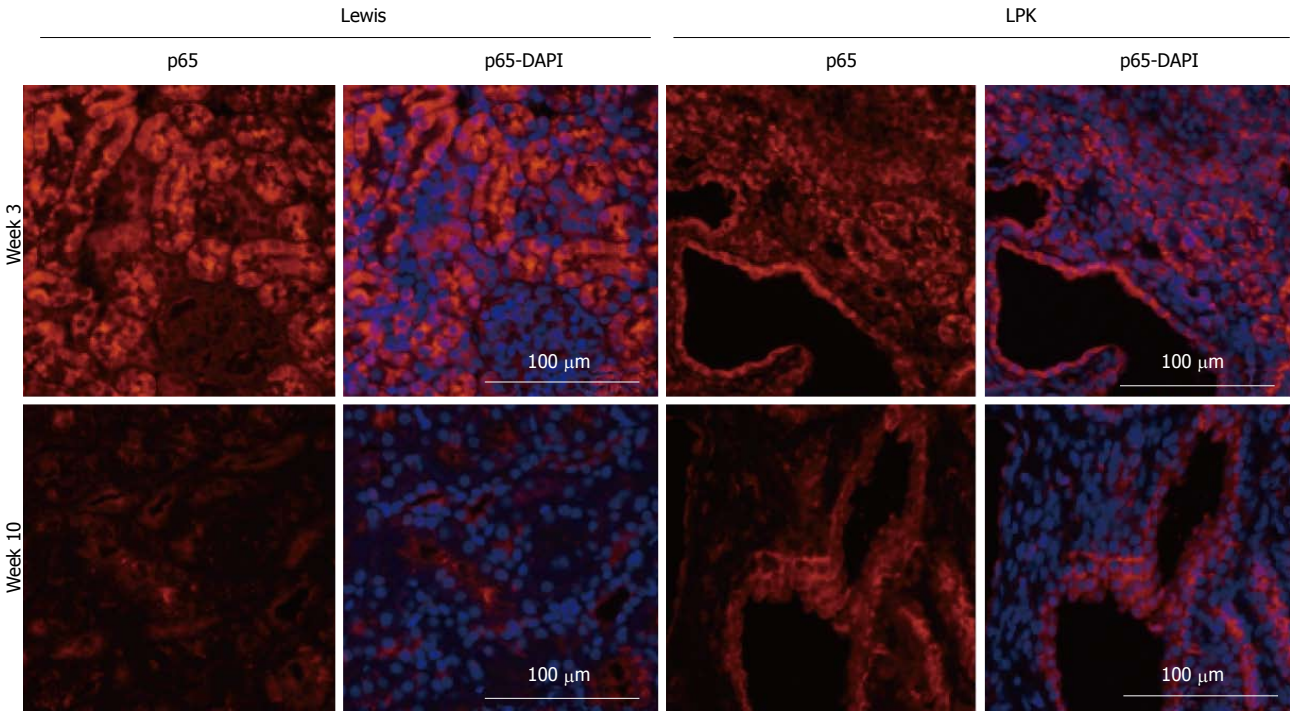



**Figure 4 Immunohistochemistry for P-p105 at weeks 3, 10, and 20 in Lewis and Lewis polycystic kidney cortex.** LPK: Lewis polycystic kidney.



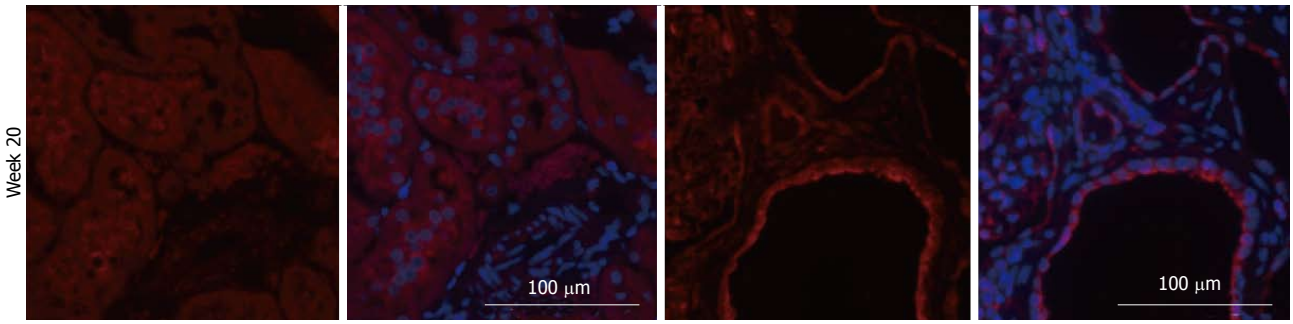



**Figure 5 Immunofluorescence staining for p65 (red) at weeks 3, 10, and 20 in Lewis and Lewis polycystic kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue). LPK: Lewis polycystic kidney.

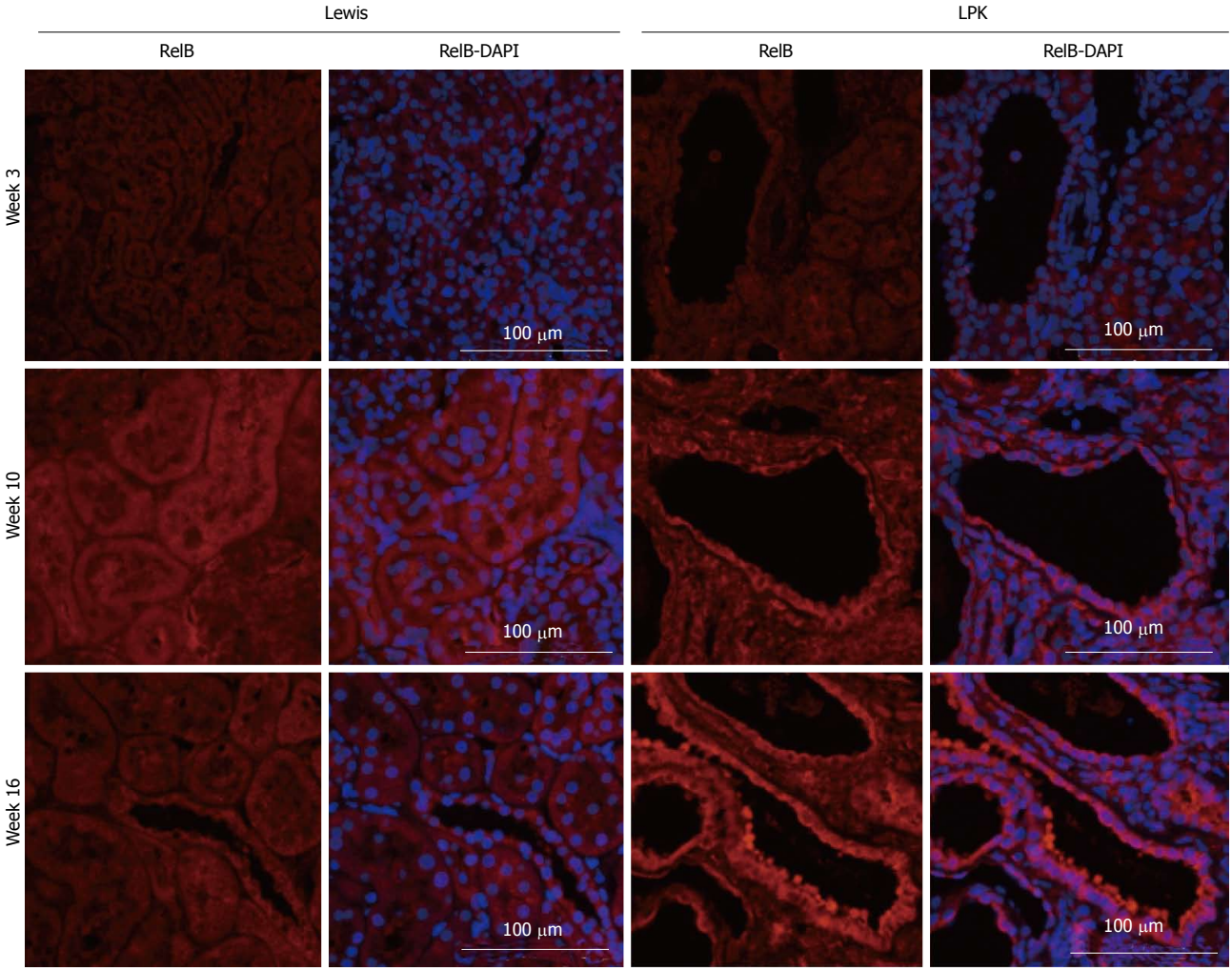



**Figure 6 Immunofluorescence staining for RelB (red) at weeks 3, 10 and 16 in Lewis and Lewis polycystic kidney cortex. Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue).** LPK: Lewis polycystic kidney.

12).

### *γ-H2AX expression in experimental and human PKD kidneys*

Previous studies in *Nek8* mice revealed that DNA damage is evident early in diseased animals with this mutation[36]. Therefore, to elucidate whether there is a relationship between NF-κB activation and DNA damage, we assessed the expression of a phosphorylated histone H2A variant, γ-H2AX, which is produced during double-strand DNA breakage[37]. A time-course study of LPK kidneys showed that γ-H2AX was strongly expressed in tubular nuclei of Lewis kidneys at week 3, and weakly expressed in later life (Figure 13). In contrast, γ-H2AX expression in the CECs of LPK kidneys was consistent throughout disease progression (Figure 13). We also found that γ-H2AX was strongly expressed in the cyst-lining cells at postnatal week 1 when NF-κB/Rel proteins were minimally

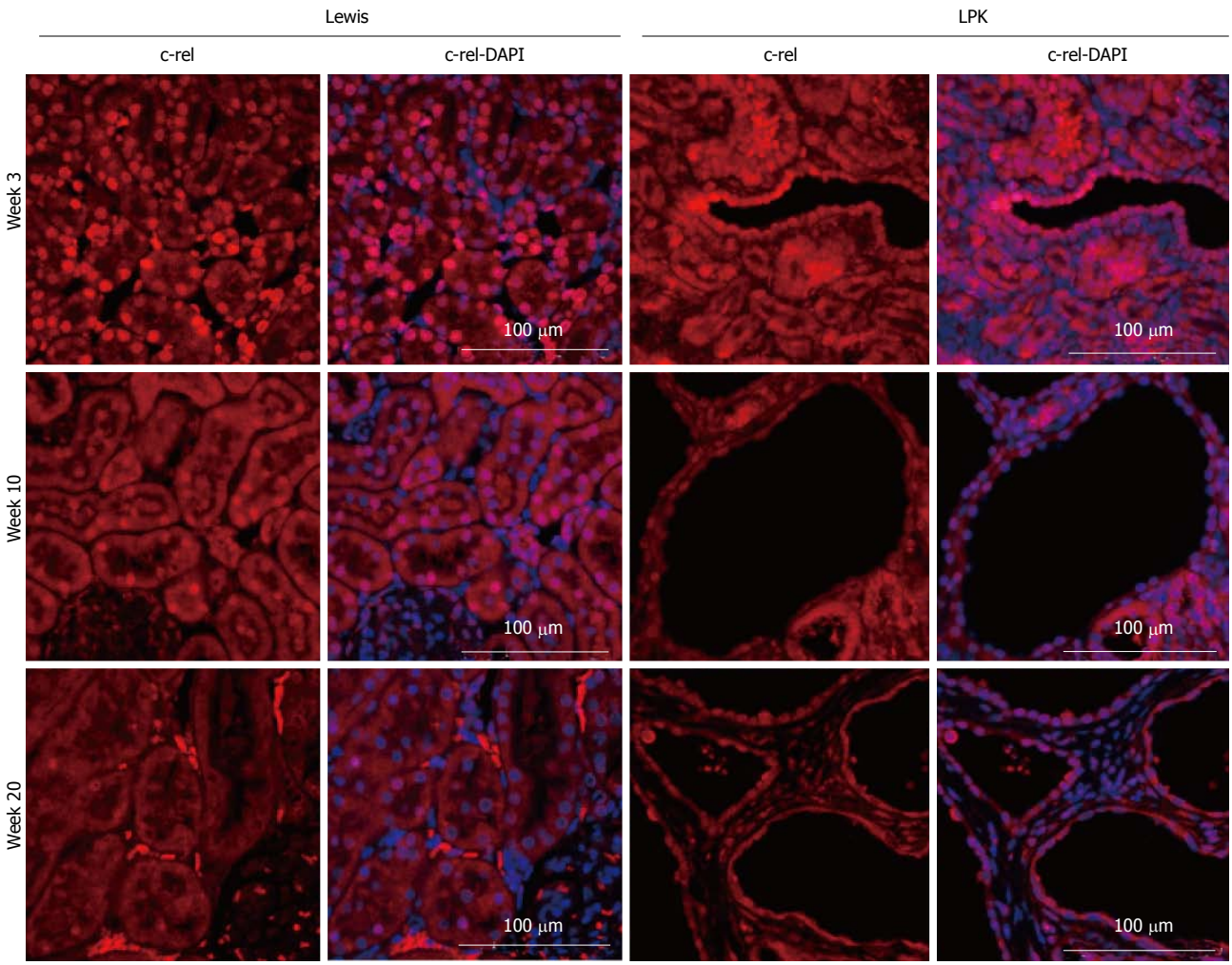
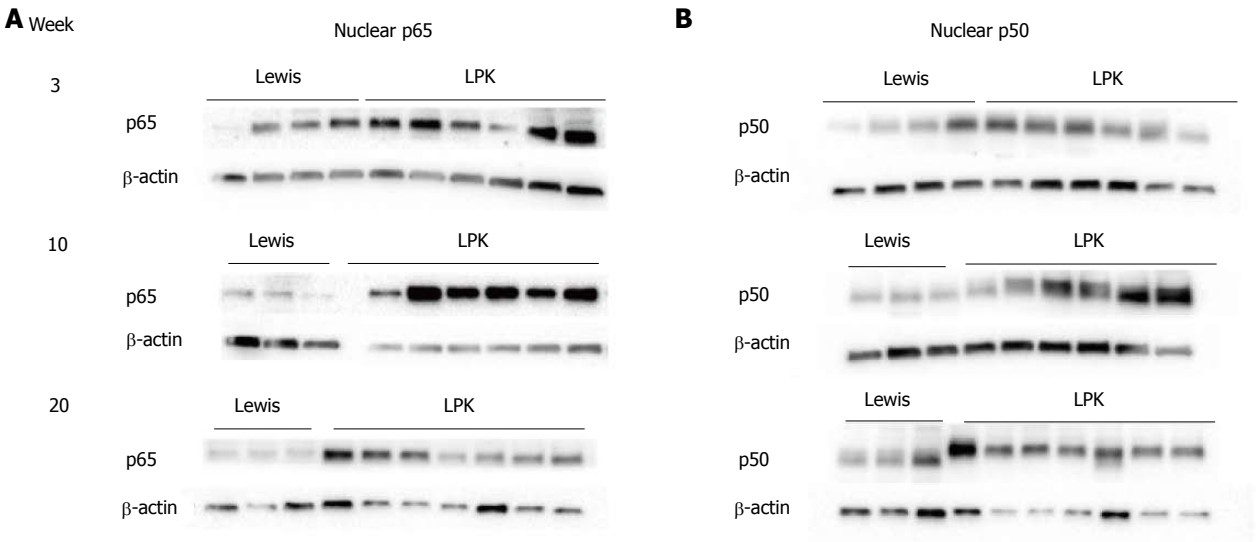



**Figure 7 Immunofluorescence staining for c-rel (red) at weeks 3, 10, and 20 in Lewis and Lewis polycystic kidney cortex. Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue).** LPK: Lewis polycystic kidney.



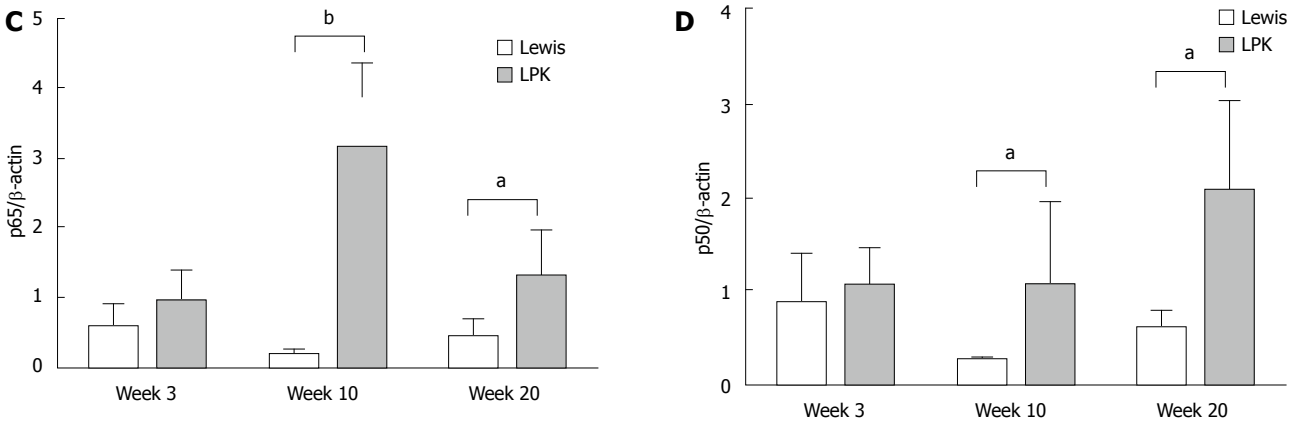



**Figure 8 Western blotting for nuclear factor-κB p65 and p50 proteins in Lewis and Lewis polycystic kidney. Immunoblotting was performed for (A) nuclear p65, and (B) nuclear p50, in Lewis and LPK kidney tissue from weeks 3, 10 and 20.** Densitometry of immunoblots was quantified for (C) p65 and (D) p50. [a]*P* < 0.05 *vs* Lewis for the corresponding timepoint; [b]*P* < 0.01 *vs* Lewis for the corresponding timepoint. LPK: Lewis polycystic kidney.

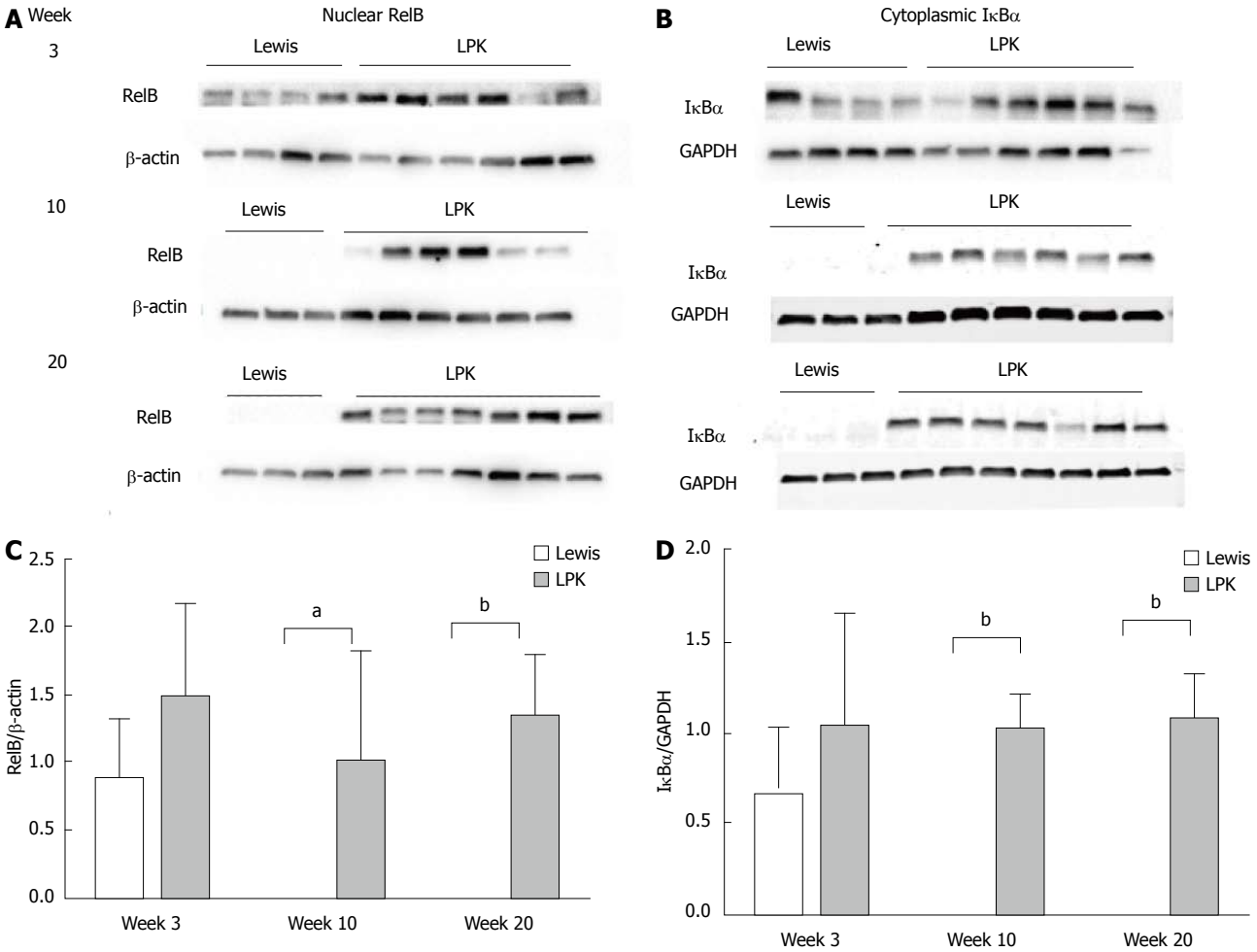



**Figure 9 Western blotting for RelB and inhibitor of κB proteins in Lewis and Lewis polycystic kidney.** Immunoblotting was performed for (A) nuclear RelB, and (B) cytoplasmic IκBα, in Lewis and LPK kidney tissue from weeks 3, 10 and 20. Densitometry of immunoblots was quantified for (C) RelB and (D) IκBα. [a]*P* < 0.05 *vs* Lewis for the corresponding timepoint; [b]*P* < 0.01 *vs* Lewis for the corresponding timepoint. LPK: Lewis polycystic kidney; IκBα: Inhibitor of kappa B.

expressed (Figure 14). In ADPKD and ARPKD, γ-H2AX was expressed in virtually all CEC nuclei (Figure 15).

## DISCUSSION

In the past decade, *in vivo* studies have demonstrated an upregulation of NF-κB proteins in PKD, but there has been limited information regarding the specific NF-κB proteins involved and their expression throughout disease progression[22,23]. The first main finding of this study is that a diverse array of NF-κB proteins, including p50, P-p105, p65, RelB and c-rel, is present in LPK

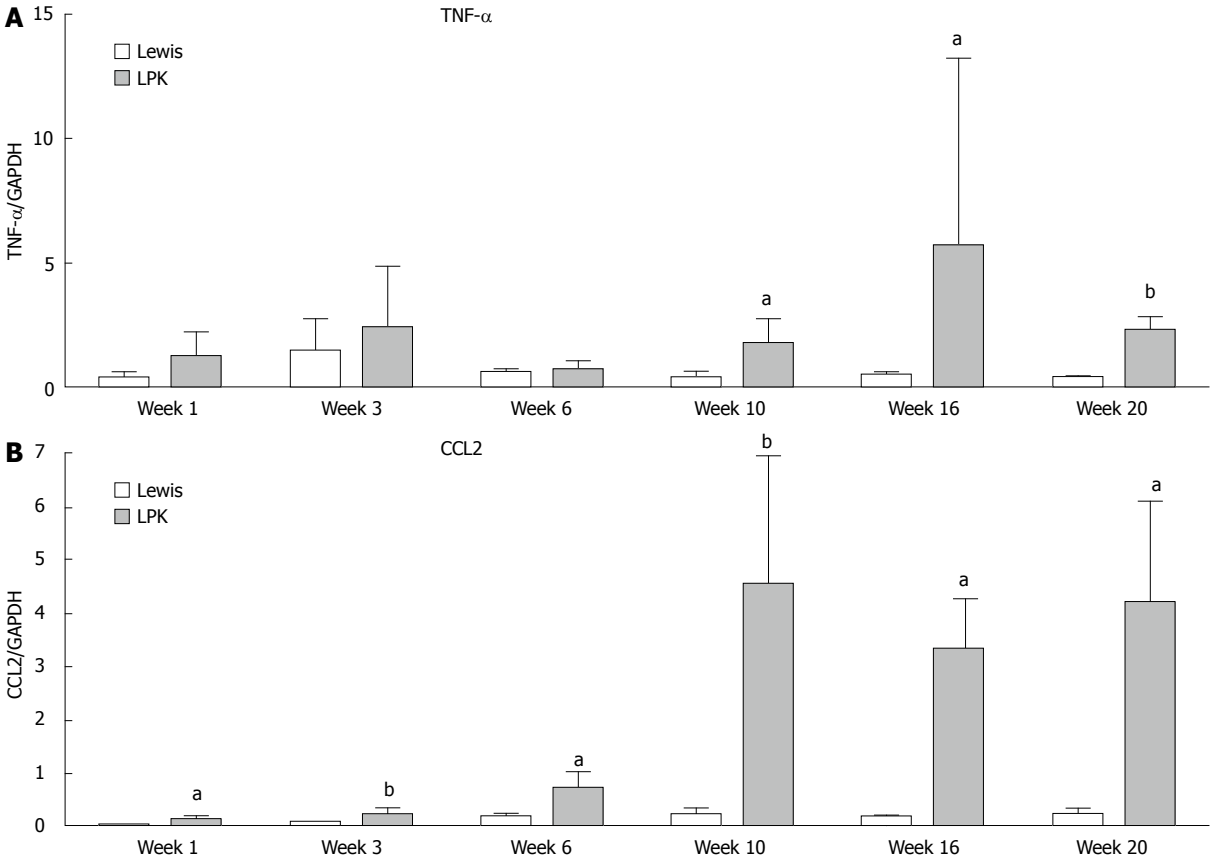



**Figure 10 Quantitative polymerase chain reaction data for (A) *TNFα* and (B) *CCL2* in Lewis and Lewis polycystic kidney tissue from weeks 1 to 20.** The mRNA expression is shown as the target gene corrected for GAPDH. [a]*P* < 0.05 *vs* Lewis for the corresponding timepoint; [b]*P* < 0.01 *vs* Lewis for the corresponding timepoint. LPK: Lewis polycystic kidney; TNF: Tumor necrosis factor.

kidneys. The localization of these NF-κB proteins to the CECs in LPK kidneys concurs with previous *in vivo* studies of *Pkd1*$^{-/-}$ and *PKD2* mice[22,23]. Notably, p65, p50 and RelB were predominantly localized to the cytoplasm rather than to the nuclei of CECs. Since NF-κB transcription factor activity is critically dependent on the translocation of NF-κB proteins to the nucleus[13], western blotting for NF-κB proteins was also performed in LPK renal nuclear extracts. This confirmed that p65, p50 and RelB proteins were present in LPK nuclei.

The second major finding was that in the LPK rat, renal NF-κB protein expression occurs early in the disease and is constitutive over time. Expression of p65 was high in both Lewis and LPK kidneys in early life, suggesting that a basal level of NF-κB activation may be required for development in the normal kidney. Indeed, NF-κB inhibition in *ex vivo* embryonic kidneys has been shown to impair ureteric bud branching, which is critical for collecting duct development[38,39]. Whereas in Lewis rats p65 expression was low from week 10 onwards, in LPK rats the high expression of p65 was sustained throughout life. Similarly, P-p105, p50, RelB and c-rel were also identified in CECs of LPK rats at all stages of disease. Western blotting revealed that nuclear p65, p50 and RelB levels were elevated in LPK compared to Lewis kidneys at week 10 and week 20.

We hypothesized that NF-κB signaling is upregulated in LPK compared to Lewis kidneys, and therefore predicted that cytoplasmic IκBα would be absent in LPK kidneys, since degradation of IκBα is necessary for NF-κB activation[13]. However, we found the converse; cytoplasmic IκBα was consistently identified in all assessed timepoints in LPK kidneys but only present in Lewis kidneys at week 3. Since cytokines can stabilize newly resynthesized IκBα, decreasing its susceptibility to further degradation[40,41], it is possible that there is a chronic upregulation of NF-κB in LPK kidneys that results in the stabilization of IκBα.

Notably, the histological data showed that NF-κB proteins are strongly expressed in the cytoplasm of LPK kidneys in early life (week 3), while immunoblotting suggested that nuclear NF-κB levels are comparable between LPK and Lewis kidneys at this timepoint and do not significantly increase in LPK compared to Lewis until weeks 10 and 20. Overall, this suggests that NF-κB proteins are present in the CEC cytoplasm at early stages of disease, but that a significant increase in NF-κB activation does not occur until later in life. However, the persistent nature of NF-κB protein expression in LPK kidneys indicates that it is a chronic, rather than transient feature of renal cystic disease in this model.

Since NF-κB regulates the transcription of *TNFα* and *CCL2*[17], and as the cytokine products of these genes are commonly found in models of PKD[8], we sought to

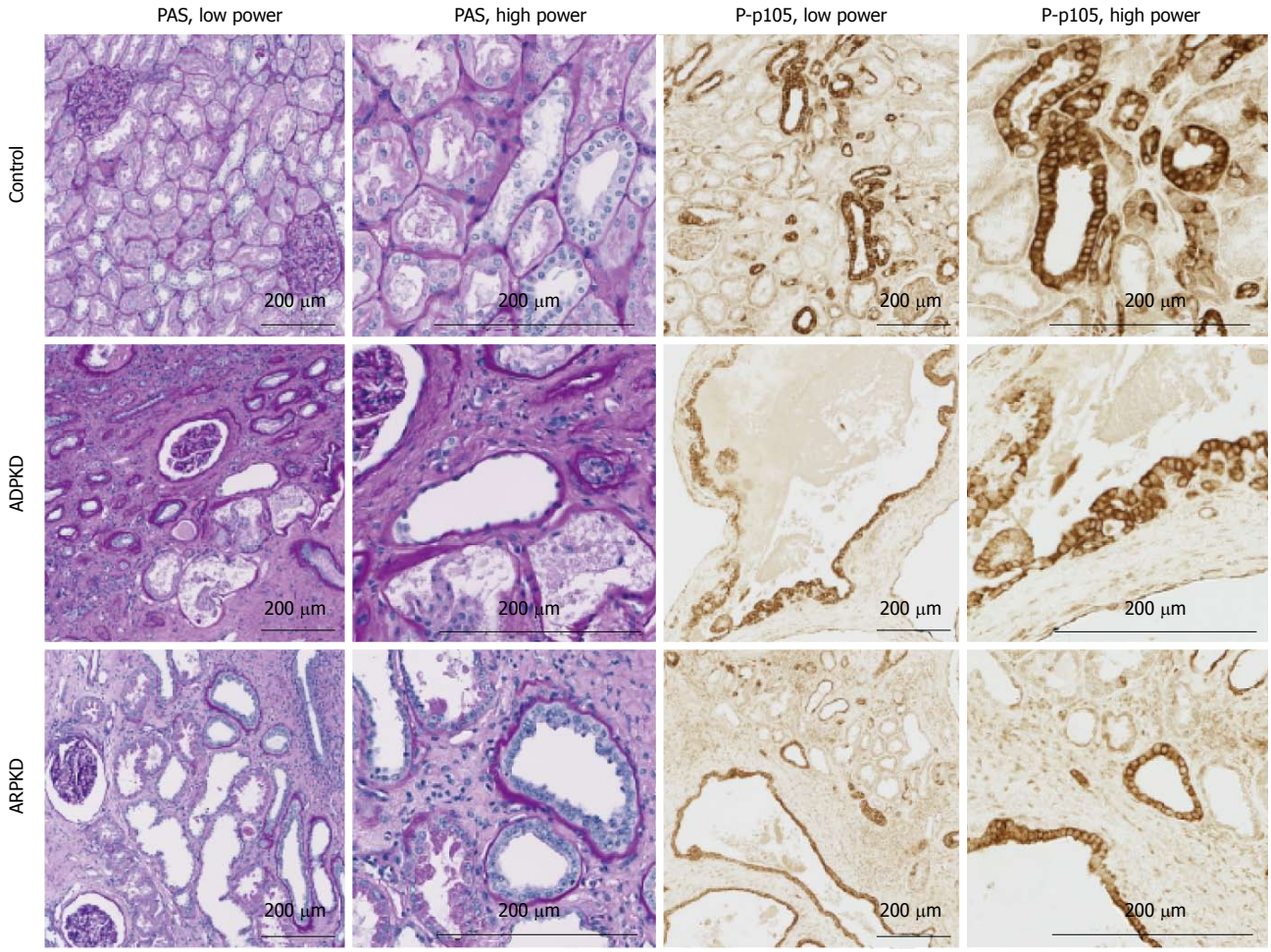



**Figure 11 Periodic Acid Schiff staining (left panels) and immunohistochemistry for P-p105 (right panels) in the cortex of human normal kidney, autosomal dominant polycystic kidney disease and autosomal recessive polycystic kidney disease.** ADPKD: Autosomal dominant polycystic kidney disease; ARPKD: Autosomal recessive polycystic kidney disease; LPK: Lewis polycystic kidney; PAS: Periodic Acid Schiff.

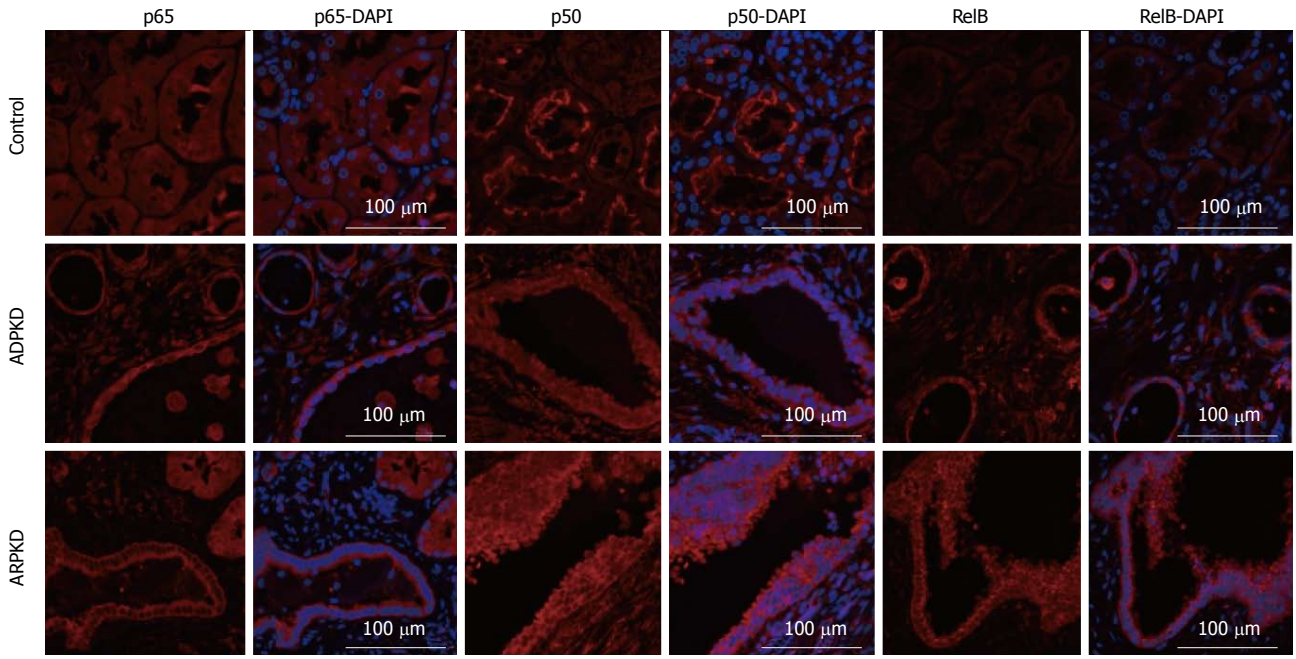



**Figure 12 Immunofluorescence staining for p65, p50 and RelB (red) in human normal kidney cortex, and in autosomal dominant polycystic kidney disease and autosomal recessive polycystic kidney disease kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue). ADPKD: Autosomal dominant polycystic kidney disease; ARPKD: Autosomal recessive polycystic kidney disease; LPK: Lewis polycystic kidney.

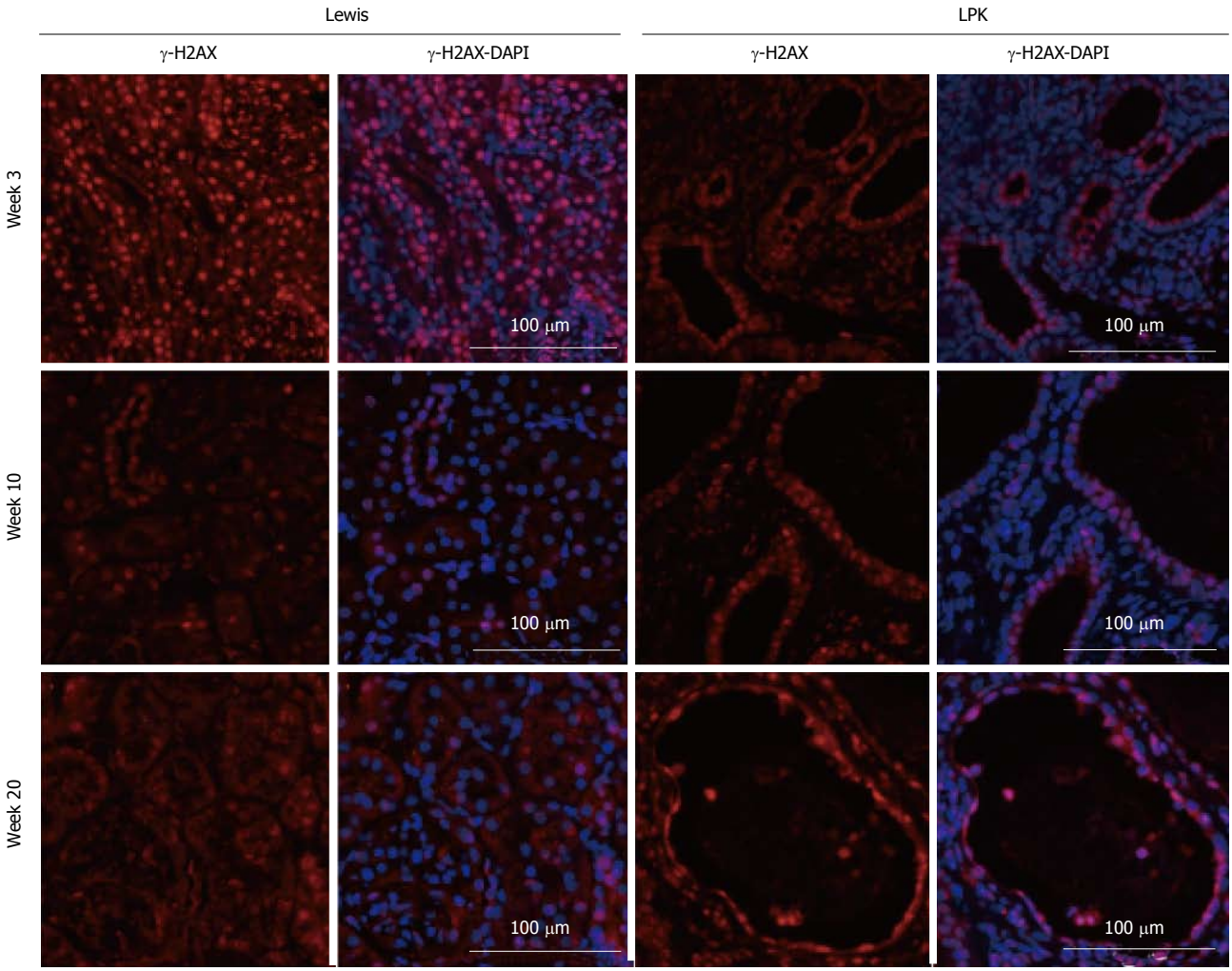



**Figure 13 Immunofluorescence staining for γ-H2AX (red) at week 3, 10 and 20 in Lewis and Lewis polycystic kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue).

determine the expression of these genes throughout the time-course of disease in LPK rats. *TNFα* expression was higher in LPK rats compared to Lewis rats, concurring with work by Nakamura *et al*[42] which found elevated *TNFα* mRNA expression in *cpk* mice compared to wild-type controls. Nakamura *et al*[42] demonstrated a steady increase in *TNFα* from early to late-stage disease. In contrast, LPK kidneys displayed a biphasic pattern of *TNFα* expression, wherein *TNFα* was upregulated at the early (week 1) and then at the late stages of disease (weeks 10 and 20). We also demonstrated an elevation in *CCL2* expression in LPK compared to Lewis at almost all timepoints, which concurs with previous findings of increased *CCL2* expression in homozygous Han:SPRD rats compared to wild-type controls[43].

To confirm the results observed in LPK rats, we also examined NF-κB expression in human cystic renal disease. Similar to LPK rats, NF-κB was localized to CECs in human ADPKD and ARPKD kidneys, and this paralleled with the findings of Park *et al*[22] which demonstrated strong staining for phosphorylated NF-κB in the CECs of ADPKD tissue. NF-κB expression was particularly intense in regions of CEC hyperplasia, suggesting that the NF-κB system regulates transcription in highly proliferating areas. In a related disorder, acquired cystic disease-associated renal cell carcinoma, phosphorylated NF-κB was identified in hyperplastic epithelial cyst-lining cells[44]. While our *in vitro* previous work found no association between the rate of proliferation and degree of NF-κB activation in human ADPKD cells[31], siRNA-induced expression of a polycystin-1 cytoplasmic terminal tail in human embryonic kidney cells led to increased NF-κB activation and proliferation[20]. Further study is therefore required to verify the relationship between NF-κB and cell proliferation in PKD. We also performed preliminary western blotting for NF-κB in normal, ADPKD and ARPKD renal tissue, and immunohistostaining for NF-κB in *Pkd2* knockout mouse kidneys, but the results were inconclusive due to small sample sizes (data not shown). Overall, the current data in LPK rats and human PKD provide a basis for future studies which may confirm the increase in NF-κB transcription activity by examining the DNA:protein binding activity of p65, p50 and RelB in human PKD tissue.

It is unclear whether NF-κB activation directly contributes to the pathogenesis of PKD, or whether it is

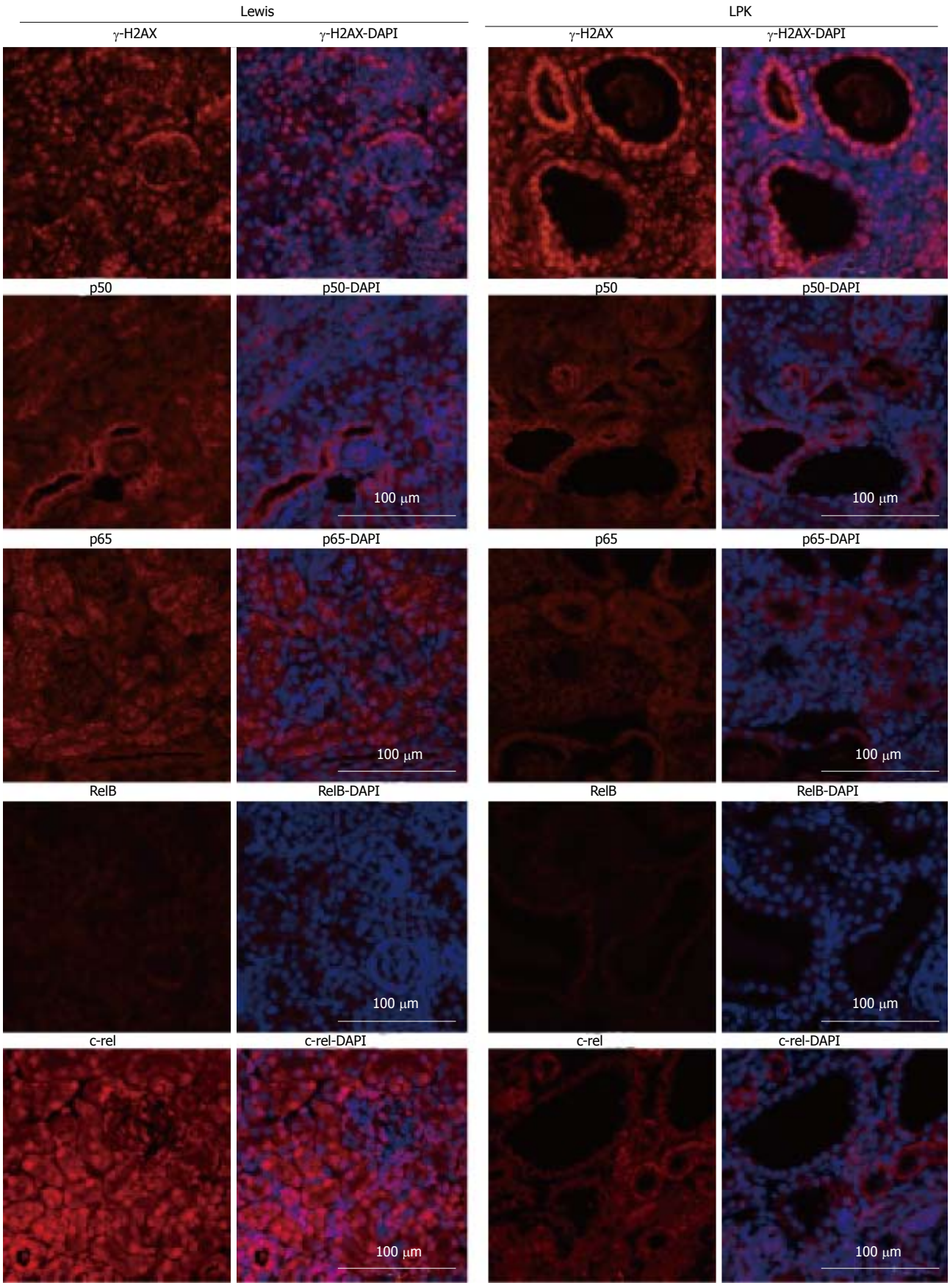



**Figure 14 Immunofluorescence staining for γ-H2AX, p50, p65, RelB and c-rel (red) at week 1 in Lewis and Lewis polycystic kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue). LPK: Lewis polycystic kidney.

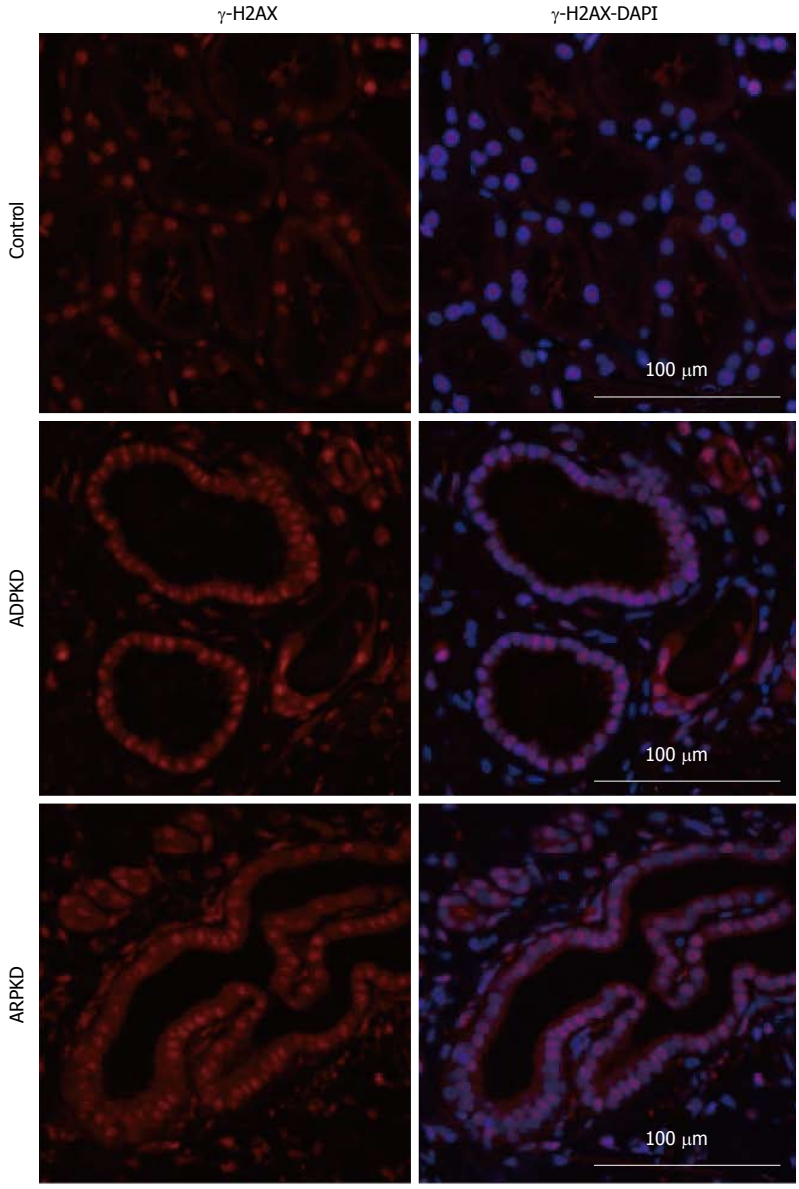



**Figure 15 Immunofluorescence staining for γ-H2AX (red) in human normal kidney cortex, and in autosomal dominant polycystic kidney disease and autosomal recessive polycystic kidney disease kidney cortex.** Also shown are corresponding DAPI-merged images with nuclei labeled using DAPI (blue). ADPKD: Autosomal dominant polycystic kidney disease; ARPKD: Autosomal recessive polycystic kidney disease.

secondary to the disease, stimulated in response to cytokines and inflammatory cells. Although our study did not directly address this question, it provided some information on the relationship between NF-κB expression and markers of PKD progression. Firstly, NF-κB proteins were observed in LPK CECs as early as week 1, and were strongly expressed at week 3, coinciding with cystic expansion and the increases in cell proliferation, inflammation and fibrosis, and continued throughout the disease time-course. The chronological order of these events may suggest that NF-κB activation contributes to cyst expansion *via* the upregulation of cell proliferation and interstitial inflammation. Qin *et al*[23] found that NF-κB inhibition decreases cyst growth in kidney explants, supporting the theory that cyst growth is NF-κB-dependent. Secondly, western blotting indicated that NF-κB protein levels were elevated in mid- to late-stage disease, coinciding with the elevations in *TNFα* and *CCL2* at weeks 10 and 20. Previous work has suggested that in *Pkd1* null mouse embryonic kidney cells, TNFα regulates its own transcription *via* the NF-κB pathway[27], and that receptor activator of NF-κB ligand (RANKL, a cytokine of the TNF family) activates the transcription of *TNFα*[45]. Taken together, these results support the notion that TNFα may contribute to NF-κB upregulation in polycystic kidneys, and furthermore suggest there may be a positive-feedback loop in which NF-κB regulates TNFα and *vice versa*. Further study is required to prove or disprove a causal relationship between NF-κB and cystic renal disease.

Our study provides preliminary data for a role of non-canonical NF-κB signaling in PKD. At weeks 10 and 20, RelB (a protein typically associated with the non-canonical pathway[13]) was absent in Lewis but present in LPK kidney extracts, suggesting that while RelB may be involved in NF-κB signaling in early normal renal development, cystic renal disease involves chronic RelB activity. Thus far, the non-canonical pathway has been associated with fewer physiological functions compared to the canonical pathway, and is mainly known for its role in B- and T-cell organogenesis[14,15]. However, studies have

suggested that non-canonical NF-κB signaling may also be involved in acute kidney injury[46] and IgA nephropathy[47]. Interestingly, RANKL is a stimulus of the non-canonical NF-κB pathway and has been implicated in NF-κB signaling in *Pkd1*$^{-/-}$ murine cells[45,46]. However since LPK kidneys also displayed elevations in p65 and *TNF*α, which are typically associated with canonical NF-κB signaling[13,16], it is likely that PKD involves a combination of canonical and non-canonical NF-κB upregulation. Future investigation of other NF-κB family members normally associated with the non-canonical pathway (*e.g.*, p52 and IKKα[16]) and NEMO (which is exclusive to the canonical pathway[14]) would be useful to further characterize NF-κB signaling in PKD.

Since DNA damage has been proposed to play a role in renal ciliopathies[48-50], we investigated the DNA damage marker, γ-H2AX, in polycystic kidneys. Our study demonstrated that γ-H2AX was strongly expressed in ADPKD and ARPKD CEC nuclei, suggesting that DNA damage may be a component of human renal cystic disease. Furthermore, γ-H2AX was apparent in CECs of LPK kidneys throughout the time-course of disease. Given that the LPK rat possesses a mutation in *Nek8*[28], and abnormalities in this gene lead to DNA double-strand breaks (DSBs) and abnormal structuring of epithelial cells in 3D spheroid cultures[36], our data add credence to the theory that *Nek8*-linked DNA damage is involved in the pathogenesis of renal ciliopathies and renal cystic diseases[48]. Interestingly, γ-H2AX was strongly expressed at week 1 in LPK kidneys, when the expression of NF-κB proteins was low (Figure 14). Since Tilstra *et al*[51] have demonstrated that NF-κB inhibition can slow the progression of DNA damage in an animal model of senescence, there is potential for NF-κB inhibition to be investigated as a strategy to ameliorate DNA injury as well as inflammation in PKD.

One limitation of this study was the small sample size and lack of genotype data for the human ADPKD and ARPKD specimens. Since in ADPKD, the *PKD2* mutation is associated with a slower progression to renal failure compared to the *PKD1* mutation[52], future studies may determine whether NF-κB activation differs according to the genotypic form and/or type of mutation (*e.g.*, missense *vs* nonsense) in ADPKD patients[53]. Also, although we demonstrated that NF-κB proteins are constitutively present in CECs of LPK rats throughout the time-course of disease, it remains unknown whether this holds true in human PKD progression. Further characterization of renal NF-κB signaling in *Pkd1* and/or *Pkd2* knockout mice (which are orthologous to human ADPKD[54]) may aid to bridge our understanding of NF-κB in human PKD. Future studies may also employ southwestern histochemistry or electrophoretic mobility shift assays (EMSA) to confirm that DNA:protein binding activity is upregulated in LPK kidneys and human PKD tissue.

In conclusion, this study found that several NF-κB proteins are expressed in the CECs of kidney tissue of LPK rats and human ADPKD and ARPKD patients. Taking into account previous studies, NF-κB upregulation has now been identified in human PKD or animal models that possess mutations in *PKD1*, *PKD2*, *PKHD1,* and *NEK8*, suggesting that it is a shared feature of cystic renal diseases and independent of genotypic variation[22,23]. Our study demonstrated that in the LPK rat, NF-κB proteins are expressed in early disease and are constitutively present throughout life. This may suggest that NF-κB inhibiting drugs need to be commenced in the early stages of PKD, in order to reduce or delay the increases in cell proliferation, interstitial inflammation and cyst volume. Our study also highlighted that RelB and non-canonical NF-κB signaling may be involved in the late stages of PKD. Although these data provide a basis for the role of NF-κB throughout disease progression in PKD, a direct causal relationship between NF-κB and PKD has not yet been proved. Future studies should address this question through cross-breeding studies of *IKK* knockout and *Pkd1*$^{-/-}$ mice, or by testing selective NF-κB inhibitors (*e.g.*, IKK inhibitors) in *in vivo* models of PKD.

## COMMENTS

### Background

The nuclear factor (NF)-κB transcription factor system is a key regulator of genes controlling inflammation and growth. The aim of this study was to determine the temporal expression of Rel/NF-κB proteins in renal tissue in polycystic kidney disease (PKD).

### Research frontiers

To date, there has been limited information regarding the particular NF-κB subunits involved in PKD and their expression throughout the time-course of disease progression. Further studies are required to elucidate whether NF-κB signaling directly contributes to cyst growth in PKD.

### Innovations and breakthroughs

This study found that NF-κB protein expression is upregulated in a rodent model of PKD, the LPK rat, and that this expression occurred early, was constitutive, and trended toward an increase over time. NF-κB upregulation was also identified in the cyst-lining cells of human autosomal dominant and recessive PKD, suggesting that this transcription factor system may regulate inflammation in cystic renal disease.

### Applications

Although this study provides promising data regarding NF-κB as a possible target for PKD, it should be noted that functional data are required to demonstrate that selective NF-κB inhibition is effective in reducing renal cystic disease in animal models. There are no selective NF-κB inhibitors approved for human use. Currently approved drugs that possess NF-κB-inhibiting properties, such as disulfiram, may be potential therapies, but require further investigation in experimental models of PKD.

### Terminology

Cystic epithelial cells: Epithelial cells that line the cyst, separating the cyst lumen from the renal interstitium. These cells rapidly proliferate, contributing to cyst expansion, and are thought respond to stimulation by intraluminal cytokines. Non-canonical NF-κB signaling: A pathway of NF-κB signaling that typically regulates the development of immune organs. Southwestern histochemistry: A technique which allows the expression of DNA-binding transcription factors to be assessed in situ. Selective NF-κB inhibition: A strategy of NF-κB inhibition by targeting specific components of NF-κB.

### Peer-review

The paper is interesting and well written. The methods are sound and the conclusions are consistent with the results.

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ORIGINAL ARTICLE

Case Control Study

# Diabetes mellitus increases the prevalence of anemia in patients with chronic kidney disease: A nested case-control study

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## Abstract

**AIM:** To compare anemia prevalence between matched chronic kidney disease (CKD) patients with and without diabetes mellitus (DM) and to assess factors associated with anemia development.

**METHODS:** This is a nested case-control study of 184 type-2 diabetic and 184 non-diabetic CKD patients from a prospectively assembled database of a Nephrology outpatient clinic, matched for gender, age and estimated glomerular filtration rate (eGFR). Prevalence of anemia (hemoglobin: Men: < 13 g/dL, women: < 12 g/dL and/or use of recombinant erythropoietin) was examined in comparison, in the total population and by CKD Stage. Univariate and multivariate logistic regression analyses were conducted to identify factors associated with anemia.

**RESULTS:** The total prevalence of anemia was higher

in diabetics (47.8% *vs* 33.2%, *P* = 0.004). Accordingly, prevalence was higher in diabetics in CKD Stage 3 (53.5% *vs* 33.1%, *P* < 0.001) and particularly in Stage 3a (60.4% *vs* 26.4%, *P* < 0.001), whereas it was non-significantly higher in Stage 4 (61.3% *vs* 48.4%; P = 0.307). Serum ferritin was higher in diabetics in total and in CKD stages, while serum iron was similar between groups. In multivariate analyses, DM (OR = 2.206, 95%CI: 1.196-4.069), CKD Stages 3a, 3b, 4 (Stage 4: OR = 12.169, 95%CI: 3.783-39.147) and serum iron (OR = 0.976, 95%CI: 0.968-0.985 per mg/dL increase) were independently associated with anemia.

**CONCLUSION:** Prevalence of anemia progressively increases with advancing stages of CKD and is higher in diabetic than matched non-diabetic CKD patients and diabetes is independently associated with anemia occurrence. Detection and treatment of anemia in diabetic CKD patients should be performed earlier than non-diabetic counterparts.

**Key words:** Anemia; Diabetes; Chronic kidney disease; Ferritin; Prevalence of anemia



**Core tip:** Anemia is an established complication of chronic kidney disease (CKD) and diabetes mellitus is proposed to further increase anemia occurrence through various mechanisms. However, a direct comparison between diabetic and non-diabetic CKD patients with regards to anemia is currently missing. This study evaluates in comparison the prevalence of anemia in carefully matched CKD patients with and without diabetes mellitus.

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## INTRODUCTION

Anemia is a major complication of chronic kidney disease (CKD) contributing to the clinical significance and the complex therapeutic approach of the uremic syndrome[1]. The prevalence of anemia (defined as serum hemoglobin levels < 130 g/L for men and < 120 g/L for women) in the general population is estimated at 7.6%, but among patients with CKD anemia is reported at least twice as prevalent, reaching 15%[2]. Anemia is generally associated with the severity of renal insufficiency, as serum hemoglobin levels and estimated glomerular filtration rate (eGFR) present an almost linear correlation[3]. Anemia commonly occurs after CKD Stage 3, with prevalence increasing from 5% in CKD Stage 1, to 75%-80% in pre-dialysis CKD Stage 5[4,5]. The main pathogenetic mechanism for the development of anemia in CKD is the impaired production of erythropoietin from kidney[6]. Iron deficiency or decreased availability, caused mainly by increased levels of hepcidin, due to inflammation accompanying chronic uremia, may constitute another important mechanism[7]. Additionally, folate and vitamin $B_{12}$ deficiency, due to malnutrition and chronic inflammation result in increased red blood cells and immature erythroblasts apoptosis[6]. Results from observational studies in pre-dialysis CKD patients suggest that anemia is associated with poor quality of life, increased hospital admissions, progression of kidney disease, and elevated mortality[8].

Diabetes mellitus (DM) is the leading cause of CKD and ESRD[9] and is proposed to elevate the risk of anemia development even in the absence of renal impairment. Anemia has been found in about 10% of patients with DM and normal kidney function[10]. In a cohort of > 9000 patients without renal disease, DM was an independent determinant of hemoglobin levels[11]. Many factors have been suggested to contribute in the pathogenesis of anemia in these patients, such as erythropoietin deficiency due to efferent sympathetic denervation of the kidney in the context of diabetic neuropathy, chronic inflammatory reaction leading to functional iron deficiency, non-selective urinary protein excretion leading to transferrin and erythropoietin loss and the use of renin-angiotensin-aldosterone system (RAAS) blockers which are central in the treatment of proteinuric diabetic nephropathy[12].

Preliminary data suggest that anemia may be more common and occurs at earlier CKD stages in diabetic patients[13]. An observational study in 1 million CKD patients of all stages indicated that prevalence of anemia in patients with DM was around 30%[14]. In another study, including patients with type 2 DM and CKD, the prevalence of anemia increased from 15% in Stage 1 to 90% in Stage 5[15]. However, epidemiologic data from a direct comparison between diabetic and non-diabetic CKD patients with regards to anemia are currently missing. On this context, the aim of this study was to examine in comparison the prevalence of anemia in matched CKD patients with and without DM and to evaluate additional factors that may contribute in anemia development.

## MATERIALS AND METHODS

### *Study design*

This is a nested case-control study in a prospectively assembled cohort of CKD patients first visiting the Nephrology Outpatient clinic of the General Hospital of Grevena, Greece between 1/01/2007 and 1/05/2015. Inclusion criteria were diagnosis of CKD and a complete dataset for the present analysis. Exclusion criteria were type 1 DM, Stage 5 CKD (eGFR < 15 mL/min per 1.73 $m^2$) or kidney transplant. In total, 184 patients with type

2 DM were included and represented the cases. After this group was formed an equal number of non-diabetic patients were selected from the same cohort by an investigator blinded to patient data apart from matching parameters to form the control group. Matching was performed for gender, age (± 5 years) and eGFR (± 5 mL/min per 1.73 $m^2$) with particular care so that both cases and controls belonged to the same CKD stage. All study procedures belonged to the routine clinical practice of the Nephrology Outpatient clinic and all patients provided informed written consent prior to study enrollment. The study protocol was approved by the Institutional Ethics Committee and all investigations were performed according to the Declaration of Helsinki (2013 amendment).

### Study data collection

For the purpose of this study, demographic and anthropometric parameters as well as cardiovascular risk factors and co-morbidities were recorded for each patient on their first outpatient visit within the aforementioned period. These included age, gender, height and weight, from which body mass index (BMI) was calculated according to the formula weight divided by height squared, as well as history of hypertension, dyslipidemia, DM, coronary heart disease, stroke, peripheral vascular disease, and cardiac arrhythmias. Moreover, data with regards to drug therapy were collected, such as medications for the treatment of DM (insulin and/or other non-insulin hypoglycaemic agents), use of medications that may affect erythropoiesis, such as oral iron supplements, recombinant erythropoietin, ACEIs or ARBs, cyclosporine, tacrolimus, *etc.,* and use of drugs interfering in the coagulation process (aspirin, clopidogrel, acenocoumarol, ticlopidine, heparin). During this visit blood samples were also acquired for the evaluation of routine hematological and biochemical parameters, including among others, serum urea, creatinine, sodium, potassium, uric acid, glucose, lipid profile and liver function tests. Patients were also instructed to perform a 24-h urine collection immediately before their next visit so that urine protein excretion would be evaluated.

### Definitions

Anemia was defined as serum hemoglobin levels < 130 g/L for men and < 120 g/L for women, according to the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines for anemia in CKD[1] and/or use of recombinant erythropoietin for known anemia. The diagnosis of DM was based on American Diabetes Association criteria[16], or on the basis of history of type 2 DM under dietary intervention or use of hypoglycaemic agents. Calculation of eGFR was performed from serum creatinine levels using the Modification of Diet in Renal Disease (MDRD) equation[17]. Definition and staging of CKD was performed according to the KDIGO 2012 guidelines[18], *i.e.,* Stage 1 CKD as eGFR ⩾ 90 mL/min per 1.73 $m^2$ plus evidence of kidney injury for more than 3 mo; Stage 2 kidney CKD as eGFR ⩾ 60 and < 90 mL/min per 1.73 $m^2$ and evidence of kidney injury; Stage 3a CKD as eGFR ⩾ 45 and < 60 mL/min per 1.73 $m^2$, Stage 3b CKD as eGFR ⩾ 30 and < 45 mL/min per 1.73 $m^2$ and Stage CKD 5 as eGFR < 15 mL/min per 1.73 $m^2$.

### Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences 21 (SPSS Inc, Chicago, IL). The Shapiro-Wilk test or Kolmogorov-Smirnov tests were used to examine the normality of distribution for quantitative variables. Continuous variables are presented as mean ± 1 SD or median range (presented in brackets) and categorical variables are described as absolute and relevant frequencies (*n*, %). $\chi^2$ test or Fisher's exact test for qualitative variables, and Student's *t*-test, Mann-Whitney test or analysis of variance (ANOVA) for quantitative variables were used for between-group comparisons. In addition, multiple logistic regression analysis was performed to evaluate the association of various studied parameters (demographic, clinical and laboratory) with anemia. Variables were tested for interactions and included in the multivariate model if $P < 0.2$ in univariate analysis. Adjusted odd ratios (OR) with 95%CI are reported. Values of $P < 0.05$ (two-tailed) were considered statistically significant.

## RESULTS

### Baseline characteristics

A total of 368 patients with CKD (Stages 2-4) were included in this study, forming two groups: The first group consisted of 184 patients with DM and CKD and the second group of 184 matched CKD patients without DM. Baseline demographic, clinical and biochemical characteristics are presented in Table 1. In each group 96 patients (52%) were male and 88 (47.8%) were female. The mean age was 75.91 ± 8.38 and 76.00 ± 9.54 years for patients with and without DM accordingly ($P = 0.908$). Patients were stratified in CKD Stages as follows: Stage 2, 14.1%; Stage 3a, 28.8%; Stage 3b, 40.2%; and Stage 4, 16.8%. With regards to the existing risk factors and comorbidities smoking habit (39.7% *vs* 16.8%; $P < 0.001$) and history of stroke (8.7% *vs* 0.5%; $P < 0.001$) were more common in diabetics. As expected, results from routine biochemical tests indicated significant differences in serum glucose levels (diabetics 8.61 ± 2.74 mmol/L, non-diabetics 5.46 ± 0.61 mmol/L; $P < 0.001$) and 24-h urine protein [diabetics 527 (59-9, 300)] mg, non-diabetics 320 (65-3, 100) mg; $P < 0.001$].

### Prevalence of anemia in total and in two study groups

As Table 2 depicts the mean hematocrit and hemoglobin levels were 39.02% ± 4.3% *vs* 40.07% ± 4.0% ($P = 0.015$) and 128.7 ± 15.6 g/L *vs* 131.9 ± 14.0 g/L ($P = 0.036$) for diabetic and the non-diabetic CKD patients respectively. Figure 1 presents the distribution of patients

**Table 1 Demographic, clinical and routine biochemical characteristics of the two study groups (patients with and without diabetes)**

| Parameter | Diabetic CKD patients | Non-diabetic CKD patients | *P* |
|---|---|---|---|
| *n* | 184 | 184 | - |
| Age (yr) | 75.91 ± 8.38 | 76.00 ± 9.54 | 0.908 |
| Gender *n* (%) | | | |
| Female | 88 (47.8) | 88 (47.8) | 1 |
| Male | 96 (52.2) | 96 (52.2) | |
| Weight (kg) | 79.78 ± 14.51 | 78.51 ± 12.58 | 0.373 |
| Height (m) | 1.67 ± 0.09 | 1.66 ± 0.08 | 0.121 |
| BMI (kg/m$^2$) | 28.34 ± 4.16 | 28.33 ± 3.26 | 0.979 |
| Urea Nitrogen (mmol/L) | 10.95 ± 5.06 | 10.90 ± 4.82 | 0.927 |
| Creatinine (μmol/L) | 136.14 ± 45.97 | 134.37 ± 46.85 | 0.826 |
| eGFR (mL/min per 1.73 m$^2$) | 43.3 ± 14.8 | 43.7 ± 14.9 | 0.778 |
| Glucose (mmol/L) | 8.61 ± 2.74 | 5.46 ± 0.61 | < 0.001 |
| 24 h urine protein excretion (mg) | 527 (59-9, 300) | 320 (65-3, 100) | < 0.001 |
| CKD Stages *n* (%) | | | |
| Stage 2 | 26 (14.1) | 26 (14.1) | 1 |
| Stage 3a | 53 (28.8) | 53 (28.8) | |
| Stage 3b | 74 (40.2) | 74 (40.2) | |
| Stage 4 | 31 (16.8) | 31 (16.8) | |
| Hypertension *n* (%) | 171 (92.9) | 175 (95.1) | 0.379 |
| Dyslipidemia *n* (%) | 103 (56) | 86 (46.7) | 0.076 |
| Coronary heart disease *n* (%) | 65 (35.3) | 60 (32.6) | 0.582 |
| Heart failure *n* (%) | 30 (16.3) | 34 (18.5) | 0.583 |
| Arrhythmia *n* (%) | 20 (10.9) | 25 (13.6) | 0.426 |
| Stroke history *n* (%) | 16 (8.7) | 1 (0.5) | < 0.001 |
| Peripheral vascular disease *n* (%) | 17 (9.2) | 13 (7.1) | 0.446 |
| Smoking *n* (%) | 73 (39.7) | 31 (16.8) | < 0.001 |

CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate.

from the two study groups over the continuum of hemoglobin levels; the distribution was in general towards lower values in patients with DM ($P$ = 0.024). Anemia was present in 149 patients accounting for 40.5% of the total population studied (Figure 2). A trend of increasing anemia prevalence was found with the progression of CKD from Stage 2 towards Stage 4, *i.e.,* Stage 2, 9.6%; Stage 3, 43.3%; Stage 4, 54.8% ($P$ < 0.001).

With regards to between-group differences, anemia was significantly more prevalent in the diabetic patient group in total (diabetics 47.8%, non-diabetics 33.2%; $P$ = 0.004). As shown in Figure 3, prevalence of anemia was higher in non-diabetics but statistically not different between the two groups in CKD Stage 2 (3.8% *vs* 15.4%, $P$ = 0.350) and thereafter higher in diabetic patients: Stage 3, 53.5% *vs* 33.1% ($P$ = 0.001); Stage 3a, 60.4% *vs* 26.4% ($P$ = 0.001); Stage 3b, 48.6% *vs* 37.8% ($P$ = 0.184); Stage 4, 61.3% *vs* 48.4% ($P$ = 0.307) for patients with and without DM accordingly.

### *Anemia-related parameters and medication use*

Results for all other anemia-related parameters are presented in Figure 2. In both groups no significant differences were noted with regards to red blood cell indices, such as mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. However, serum ferritin levels were significantly higher in patients with DM both in total and in all CKD stages, while serum iron levels were equal between groups, with the exception of CKD Stage 2, in which patients with DM had 15.39 (6.09-23.63) μmol/L and patients without DM 12.35 (2.15-23.09) μmol/L ($P$ = 0.027).

Use of recombinant erythropoietin was similar between the two study groups in total (diabetics 8.2%, non-diabetics 8.7%; $P$ = 0.851) and in CKD stages separately and the use of oral iron supplementary therapy was similar (diabetics 14.7%, non-diabetics 9.8%, $P$ = 0.152). Regarding other medications that may interfere with development of anemia, use of RAAS-blockers did not differ significantly between diabetics (65.8%) and non-diabetics (70.1%, $P$ = 0.372) in total and in CKD stages respectively. Finally, the use of drugs interfering in the coagulation process was higher for patients with DM in total (diabetics 46.7%, non-diabetics 35.9%; $P$ = 0.034), but differences were not significant between the two groups in CKD stages.

### *Factors associated with anemia*

Univariate and multivariate regression analyses in the total population studied is presented in Table 3. Anemia was the dependent variable, while several demographic, clinical and laboratory factors that can interfere with development of anemia were the independent variables. Diabetes was an independent factor for anemia occurrence in the total population (OR = 2.206, 95%CI: 1.196-4.069). Advancing stage of CKD was associated with progressively increasing risk for anemia development both in univariate and multivariate analysis; *i.e.,* Stage 3a (OR = 6.068, 95%CI: 2.112-17.430), Stage 3b

**Table 2 Comparisons between the two study groups for anemia-related parameters in total study population and by chronic kidney disease stages (statistically significant *P* values are indicated in bold)**

| Parameter | Total study population | *P* | Stage 2 | *P* | Stage 3 | *P* | Stage 3a | *P* | Stage 3b | *P* | Stage 4 | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hematocrit (%) | | | | | | | | | | | | |
| Diabetics | 39.02 ± 4.30 | 0.015 | 42.27 ± 4.79 | 0.324 | 38.69 ± 3.97 | 0.001 | 38.92 ± 4.08 | < 0.001 | 38.53 ± 3.91 | 0.278 | 37.63 ± 4.02 | 0.687 |
| Non-diabetics | 40.07 ± 4 | | 41.18 ± 2.83 | | 40.34 ± 4.09 | | 41.93 ± 4.10 | | 39.21 ± 3.71 | | 38.04 ± 3.9 | |
| Hemoglobin (g/L) | | | | | | | | | | | | |
| Diabetics | 128.7 ± 15.6 | 0.036 | 141.8 ± 17.4 | 0.21 | 127.4 ± 14.1 | 0.003 | 128.2 ± 14.8 | 0.001 | 126.9 ± 13.6 | 0.383 | 122.7 ± 14.1 | 0.58 |
| Non-diabetics | 131.9 ± 14.0 | | 136.7 ± 10.9 | | 132.7 ± 14.1 | | 138.2 ± 14.0 | | 128.8 ± 13.0 | | 124.7 ± 13.5 | |
| MCV (fL) | | | | | | | | | | | | |
| Diabetics | 87.62 ± 6.99 | 0.739 | 87.76 ± 74.2 | 0.536 | 87.70 ± 7.49 | 0.633 | 87.78 ± 5.88 | 0.81 | 87.65 ± 8.5 | 0.457 | 87.11 ± 6.86 | 0.527 |
| Non-diabetics | 87.9 ± 6.99 | | 86 ± 13.7 | | 88.20 ± 8.83 | | 87.45 ± 8.34 | | 88.73 ± 9.17 | | 88.29 ± 7.68 | |
| MCH (pg/cell) | | | | | | | | | | | | |
| Diabetics | 29.91 ± 5.08 | 0.748 | 29.53 ± 2.21 | 0.684 | 30.28 ± 5.89 | 0.494 | 29.52 ± 2.24 | 0.792 | 30.82 ± 7.45 | 0.526 | 28.76 ± 2.42 | 0.415 |
| Non-diabetics | 29.78 ± 2.64 | | 29.83 ± 3 | | 29.89 ± 2.55 | | 29.4 ± 2.75 | | 30.25 ± 2.35 | | 29.3 ± 2.73 | |
| MCHC (g/L) | | | | | | | | | | | | |
| Diabetics | 323.8 ± 17.6 | 0.523 | 333.6 ± 12.0 | 0.03 | 321.6 ± 19.0 | 0.362 | 323.0 ± 21.7 | 0.982 | 320.6 ± 16.9 | 0.174 | 324.8 ± 11.6 | 0.282 |
| Non-diabetics | 322.6 ± 20.1 | | 319.5 ± 29.5 | | 323.7 ± 18.4 | | 322.9 ± 20.9 | | 324.3 ± 16.4 | | 320.3 ± 19.7 | |
| Serum iron (μmol/L) | | | | | | | | | | | | |
| Diabetics | 12.35 (1.61-35.73) | 0.783 | 2.75 (1.09-4.23) | 0.027 | 12.17 (1.61-35.73) | 0.351 | 12.71 (1.61-28.28) | 0.86 | 11.01 (2.15-35.73) | 0.194 | 10.92 (3.83-20.23) | 0.559 |
| Non-diabetics | 12.53 (2.69-27.03) | | 2.21 (0.38-4.13) | | 12.53 (2.69-27.03) | | 12.35 (4.47-23.27) | | 12.71 (2.69-27.03) | | 11.99 (3.94-23.81) | |
| Ferritin (ng/mL) | | | | | | | | | | | | |
| Diabetics | 200 (17.3-1048.7) | < 0.001 | 230.3 (62.9-570.7) | 0.01 | 175.3 (17.3-1048.7) | 0.003 | 175.3 (23.4-1048.7) | 0.013 | 175.3 (7.7-1015.6) | 0.061 | 220.2 (25.6-867.3) | 0.011 |
| Non-diabetics | 148.3 (7.2-993.2) | | 155.1 (78.6-435.9) | | 148.3 (22.5-993.2) | | 155.1 (22.5-294.4) | | 143.8 (26.9-993.2) | | 143.8 (7.2-441.4) | |
| 24 h urine protein Excretion (mg) | | | | | | | | | | | | |
| Diabetics | 527 (59-9300) | < 0.001 | 283 (68-5100) | 0.126 | 530 (59-9300) | < 0.001 | 545 (129-1700) | < 0.001 | 525 (59-9300) | < 0.001 | 670 (95-3800) | 0.647 |
| Non-diabetics | 320 (65-3100) | | 245 (110-780) | | 300 (65-3100) | | 250 (65-1500) | | 375 (104-3100) | | 560 (117-3100) | |
| Smoking (*n*, %) | | | | | | | | | | | | |
| Diabetics | 73 (39.7) | < 0.001 | 10 (38.5) | 0.375 | 57 (44.9) | < 0.001 | 32 (60.4) | 0.011 | 25 (33.8) | < 0.001 | 6 (19.4) | 0.255 |
| Non-diabetics | 31 (16.8) | | 7 (26.9) | | 22 (17.3) | | 19 (35.8) | | 3 (4.1) | | 2 (6.5) | |
| Use of erythropoietin (*n*, %) | | | | | | | | | | | | |
| Diabetics | 15 (8.2) | 0.851 | 0 (0) | n/a | 9 (7.1) | 0.271 | 4 (7.5) | 0.118 | 5 (6.8) | 1 | 6 (80.6) | 0.155 |
| Non-diabetics | 16 (8.7) | | 0 (0) | | 5 (3.9) | | 0 (0) | | 5 (6.8) | | 11 (35.5) | |
| Iron supplements therapy (*n*, %) | | | | | | | | | | | | |
| Diabetics | 27 (14.7) | 0.152 | 1 (3.8) | 1 | 19 (15) | 0.076 | 8 (15.1) | 0.111 | 11 (14.9) | 0.314 | 7 (22.6) | 1 |
| Non-diabetics | 18 (9.8) | | 1 (3.8) | | 10 (7.9) | | 3 (5.7) | | 7 (9.5) | | 7 (22.6) | |
| ACEIs/ARBs (*n*, %) | | | | | | | | | | | | |
| Diabetics | 121 (65.8) | 0.372 | 22 (84.6) | 1 | 88 (69.3) | 0.784 | 38 (71.7) | 0.831 | 50 (67.6) | 0.592 | 11 (35.5) | 0.075 |
| Non-diabetics | 129 (70.1) | | 21 (80.8) | | 90 (70.9) | | 37 (69.8) | | 53 (71.6) | | 18 (58.1) | |
| Antiplatelet/anticoagulant drugs (*n*, %) | | | | | | | | | | | | |
| Diabetics | 86 (46.7) | 0.034 | 9 (34.6) | 0.199 | 62 (48.8) | 0.165 | 27 (50.9) | 0.171 | 35 (47.3) | 0.508 | 15 (48.4) | 0.303 |
| Non-diabetics | 66 (35.9) | | 4 (15.4) | | 51 (40.2) | | 20 (37.7) | | 31 (41.9) | | 11 (35.5) | |

ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blocker; CKD: Chronic kidney disease; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; n/a: Not applicable.

(OR = 7.499, 95%CI: 2.604-21.597), Stage 4 (OR = 12.169, 95%CI: 3.783-39.147). Serum iron levels were also associated with occurrence of anemia (OR = 0.976, 95%CI: 0.968-0.985 per mg/dL increase). Interestingly, female gender was associated with decreased risk for anemia occurrence (OR = 0.389, 95%CI: 0.224-0.675), but this may be related to the lower threshold of hemoglobin for females in the definition used. With regards to other existing comorbidities no significant correlations were observed. Similarly, use of RAAS-blockers and antiplatelet or anticoagulant drugs, as well as the degree of 24-h urine protein excretion were not found to be associated with the development of anemia.

## DISCUSSION

This study examined in comparison the prevalence of anemia in matched CKD patients with and without DM and further aimed to evaluate the possible association of demographic, clinical and laboratory factors with the development of anemia. The overall prevalence of anemia in the population studied was high (40.5%), while the prevalence in patients with DM was about 15% higher than that in non-diabetic counterparts

**Table 3 Univariate and multivariate regression analysis for occurrence of anemia (defined as serum hemoglobin levels < 130 g/L for men and < 120 g/L for women and/or use of recombinant erythropoietin) in the total studied population**

| Parameter | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | Unadjusted odds ratio (95%CI) | *P* | Adjusted odds ratio (95%CI) | *P* |
| BMI Groups | | | | |
| Normal (18.5-25) | Reference group | | | |
| Underweight (< 18.5) | 0.545 (0.046-6.443) | 0.63 | | |
| Overweight (25-30) | 0.714 (0.377-1.350) | 0.3 | | |
| Obese (> 30) | 0.708 (0.348-1.442) | 0.342 | | |
| Age | | | | |
| < 75 yr | Reference group | | Reference group | |
| ⩾ 75 yr | 1.623 (1.028-2.564) | 0.038 | 1.198 (0.694-2.069) | 0.517 |
| Gender | | | | |
| Male | Reference group | | Reference group | |
| Female | 0.546 (0.357-0.833) | 0.005 | 0.389 (0.224-0.675) | 0.001 |
| CKD Stages | | | | |
| Stage 2 | Reference group | | Reference group | |
| Stage 3a | 7.207 (2.656-19.566) | < 0.001 | 6.068 (2.112-17.430) | 0.001 |
| Stage 3b | 7.162 (2.694-19.038) | < 0.001 | 7.499 (2.604-21.597) | < 0.001 |
| Stage 4 | 11.414 (3.999-32.582) | < 0.001 | 12.169 (3.783-39.147) | < 0.001 |
| Diabetes | 1.848 (1.212-2.818) | 0.004 | 2.206 (1.196-4.069) | 0.011 |
| Hypertension | 0.663 (0.280-1.573) | 0.351 | | |
| Dyslipidemia | 0.745 (0.491-1.130) | 0.166 | 0.659 (0.404-1.074) | 0.094 |
| Coronary heart disease | 1.446 (0.934-2.239) | 0.098 | 1.048 (0.506-1.960) | 0.883 |
| Heart failure | 1.725 (1.003-2.967) | 0.049 | 1.228 (0.628-2.398) | 0.548 |
| Arrhythmia | 0.788 (0.412-1.509) | 0.472 | | |
| Smoking | 1.051 (0.662-1.667) | 0.834 | | |
| Serum glucose levels (per mg/dL increase) | 1.006 (1.002 to 1.011) | 0.009 | 0.999 (0.992-1.005) | 0.736 |
| Serum iron (per mg/dL increase) | 0.978 (0.970-0.986) | < 0.001 | 0.976 (0.968-0.985) | < 0.001 |
| Ferritin (per ng/mL increase) | 0.998 (0.995-1.001) | 0.209 | | |
| 24 h urine protein excretion (per mg increase) | 1.000 (1.000-1.003) | 0.146 | 1.000 (1.000-1.001) | 0.772 |
| ACEIs/ARBs | 0.690 (0.443-1.075) | 0.101 | 0.963 (0.565-1.641) | 0.888 |
| Antiplatelet/anticoagulant drugs | 1.413 (0.927-2.156) | 0.108 | 1.161 (0.669-2.015) | 0.595 |

ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blocker; CKD: Chronic kidney disease; BMI: Body mass index.

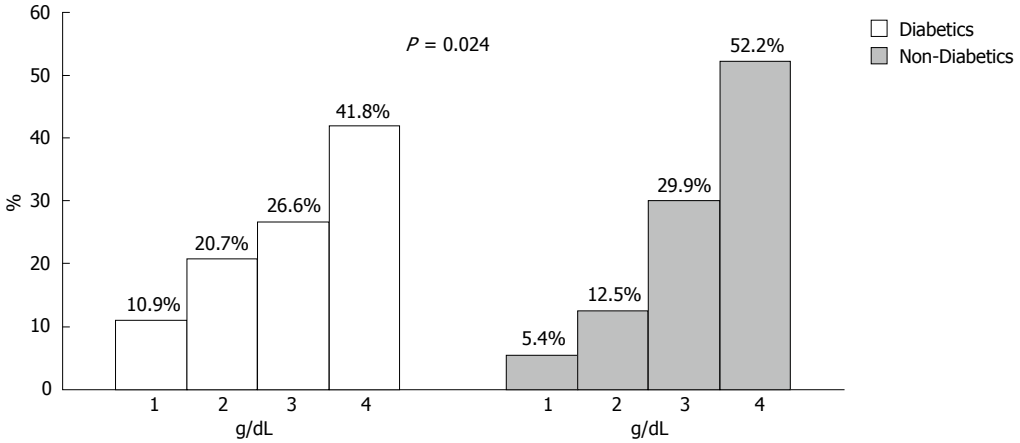


**Figure 1 Distribution of serum hemoglobin levels in diabetic and non-diabetic patients.** Percentages indicate the % of patients with hemoglobin levels within each depicted category of hemoglobin levels. 1: ⩽ 11; 2: > 11-⩽12; 3: > 12-<13; 4: ⩾ 13.

(47.8% *vs* 33.2%). With the exception of Stage 2, where the overall prevalence was low (9.5%), anemia was more prevalent in the diabetic patients group in the rest CKD stages, with the difference between groups being particularly large at CKD Stage 3a, where diabetic patients had more than two times higher anemia occurrence (60.4% *vs* 26.4%). Serum ferritin levels, but not iron, was higher in diabetic than in non-diabetic patients in all stages; as the former also had higher rates of anemia, increased ferritin may mirror its role as an acute phase reactant, signifying higher subclinical inflammation in diabetic patients. In multivariate analyses, among a wide set of demographic, co-morbid, laboratory and medication parameters studied presence of DM, CKD Stages 3a, 3b and 4 and serum iron levels were independently associated with anemia occurrence.

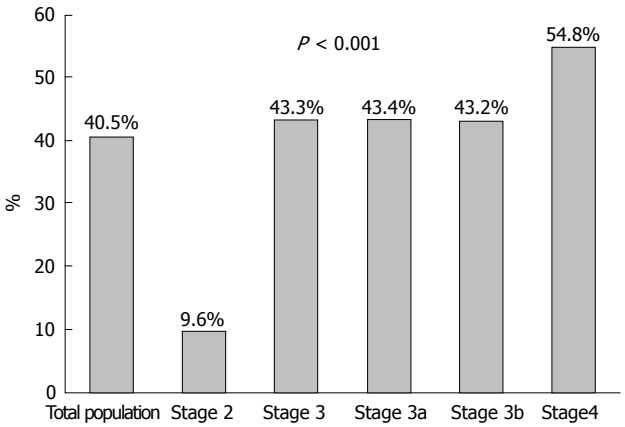


**Figure 2 Prevalence anemia in total studied population and in chronic kidney disease Stage 2, 3, 3a, 3b and 4.**

Anemia is an established complication of CKD and is per se associated with the severity of renal insufficiency, mostly due to impaired production of endogenous erythropoietin and true deficiency or decreased availability of serum iron[7,8]. This study further supports this principle, as our results indicated progressing increase in prevalence of anemia with the progression of CKD from Stage 2 (9.6%) to Stage 3 (43.3%) and Stage 4 (54.8%). Moreover, advancing stage of CKD was independently associated with progressively higher OR levels for the development of anemia from CKD Stage 3a (O R= 6.068), CKD Stage 3b (OR = 7.499) and CKD Stage 4 (OR = 12.169). These results are in accordance to the National Health and Nutrition Examination Survey (NHANES) in which prevalence of anemia was 5% in patients with CKD Stage 1 and reached progressively 80% in pre-dialysis Stages 4-5 patients[4]. Similarly, in another cross-sectional study of 5000 individuals with CKD, prevalence of anemia in overall was 48% and was associated with eGFR deterioration as it increased from 27% to 75% with the progression from CKD Stage 2 to CKD Stage 5[5].

Previous indirect data suggested that diabetic patients with CKD may exhibit higher rates of anemia in relation to patients without DM. Patients with type 2 DM may experience anemia even in the absence of nephropathy, as indicated by a previous observational study, in which 16% of the individuals who had type 2 DM but no CKD developed anemia in a 7-year follow up[10]. In a cross-sectional study of > 1 million patients with CKD of Stages 1-5, in which 5% were diabetics, prevalence of anemia was twice as high in diabetics (30% *vs* 15%) in total, but prevalence in each CKD stage with regards to diabetes presence was not evaluated[14]. In a cohort study of type-2 diabetic CKD patients, prevalence of anemia was 15% in Stage 1, 25% in Stage 2, 50% in Stage 3 and 90% in Stages 4-5[15]. Two other studies have associated DM with increased occurrence of anemia in CKD. The first, including almost 5400 individuals with CKD, of whom 27% had DM, indicated an overall prevalence of anemia 11.6% among diabetics, with its frequency increasing about 45% from CKD Stage 1 to Stage 5[19]. The second studied 468 unmatched CKD patients of whom 44% were type 1 or type 2 diabetics and prevalence of anemia in patients with DM was 17% in CKD Stages 1-2, 51% in CKD Stage 3 and 59% in CKD Stages 4-5, while DM was associated with a significant fourfold increase in risk of anemia in the regression analysis[20]. In contrast, results from the Pre-dialysis Survey of Anemia Management Study indicated no significant differences between patients with and without DM regarding the correlation of serum hemoglobin levels and creatinine clearance rate[21]. Our study further clarifies this issue, showing higher prevalence of anemia in diabetic than carefully matched non-diabetic CKD patients, particularly in Stage 3a, where the majority of individuals with CKD belongs.

As discussed above, several mechanisms promoting anemia in diabetic individuals have been previously described. Erythropoietin deficiency due to efferent sympathetic denervation of the kidney as a result of diabetic neuropathy, subclinical inflammation leading to functional iron deficiency through increased hepcidin levels, increased non-selective proteinuria excretion resulting in transferrin and erythropoietin loss, increased red blood cell destruction because of disorders in the cellular structure caused by DM and advanced glycation end products (AGEs) possibly decreasing erythrocyte lifespan are among them[11-13,22,23]. Further, increased use of RAAS-blockers in diabetic patients, may promote anemia occurrence through inhibition of the physiologic erythropoietic action of angiotensin Ⅱ[24]. In our study, proteinuria was significantly higher in diabetic patients, but it did not display significant associations with anemia in multivariate analysis. Further, the use of RAAS-blockers was practically equal between the two groups, thus it could not significantly affect the results; use of ACEIs or ARBs was also not associated with anemia in multivariate analysis.

A role of chronic inflammation affecting anemia in DM is also proposed. Recent findings suggest that diabetic patients have higher ferritin and hepcidin levels than matched non-diabetic individuals[25]. Levels of ferritin as a marker of inflammation and hepcidin were shown to correlate strongly in various populations including patients with DM[26] and CKD of various types[27]. Increased hepcidin following subclinical inflammation has also been observed in obese individuals[28]. Hepcidin is the key factor causing functional iron deficiency reducing the efflux of recycled iron from both splenic and hepatic macrophages and also inhibits iron absorption from the gut; the overall reduction of iron available for erythropoiesis leads to anemia[28]. Our findings support this mechanism of chronic inflammation as ferritin levels were significantly higher in diabetics in overall (200.0 pmol/L *vs* 148.3 pmol/L; $P < 0.001$) and in almost every CKD stage. In addition, although an increase in serum iron was associated with less anemia occurrence in multivariate analysis, ferritin levels displayed no significant associations, a finding

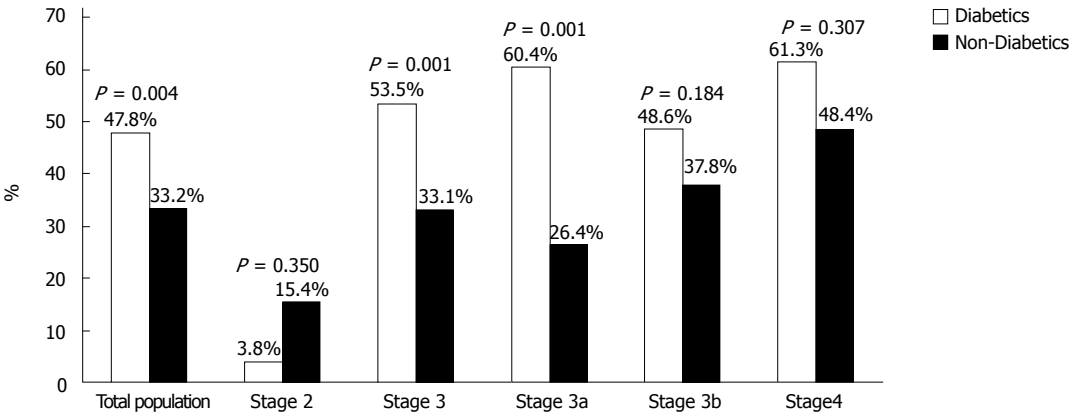


**Figure 3 Prevalence of anemia in diabetic and non-diabetic patients in total and in chronic kidney disease Stage 2, 3, 3a, 3b and 4.**

suggesting that ferritin could not be considered as a marker of iron stores. Further examination of this pathway including measurement of hepcidin levels could be useful.

This study has methodologic strengths. Although prevalence of anemia in CKD and DM has been examined previously, a direct comparison in patients with and without DM in CKD, to the best of our knowledge, was absent. Apart from the careful matching of individuals to form the two study groups, the capture of several factors that may theoretically affect the development of anemia in DM and a careful multiple logistic regression analysis further strengthen our results. However, there are also some limitations. This is an observational study, thus definite cause and effect associations cannot be established. The use of a unique hemoglobin measurement to determine the diagnosis of anemia may have misclassified some individuals. Finally, observed frequencies and significance levels in some comparisons may have been affected to an extent by the relatively small sample sizes in some of the subgroup analyses.

In conclusion, this study has confirmed that anemia is common in CKD outpatients and increases steadily with advancing Stages of CKD. Furthermore, the prevalence of anemia is higher in diabetic patients with CKD compared to matched non-diabetic counterparts. The difference between diabetic and non-diabetic patients with CKD was more prominent in CKD Stage 3a, where the majority of individuals with CKD belongs. Subclinical inflammation in diabetic patients with moderate CKD may be the most important underlying factor for this association, as indicated by increased ferritin levels in diabetics in our study. As anemia is associated with significant morbidity and mortality, both detection and treatment of anemia in diabetic CKD patients should be performed earlier than in non-diabetic counterparts.

## COMMENTS

### Background

Anemia is a major complication of chronic kidney disease (CKD) and diabetes mellitus (DM) is proposed to elevate the risk of anemia development. However, epidemiologic data from a direct comparison between diabetic and non-diabetic CKD patients with regards to anemia are currently missing.

### Research frontiers

Prevalence of anemia has been extensively studied in patients with CKD. However, current evidence about the role of DM in anemia development in CKD derive only from observational studies in CKD population in which diabetics constitute only a small proportion. DM has been found to further elevate the prevalence of anemia in CKD, but not in all studies. On this context, a study examining in comparison the prevalence of anemia in matched CKD diabetic and non-diabetic patients would further clarify the role of DM in anemia development.

### Innovations and breakthroughs

This study is the first to evaluate prevalence of anemia with a case control design in carefully matched diabetic and non-diabetic CKD patients.

### Applications

Both detection and treatment of anemia in diabetic CKD patients should be performed earlier than in non-diabetics, in order to prevent anemia-associated complications.

### Peer-review

The study deals with a common issue in clinical practice; (*i.e.*, diabetic patients with moderate CKD often appear with low Hb levels for their eGFR levels and have already been investigated for anemia from internists or hematologists for years with no results). Although there are some previous data pointing to the fact that anemia (among many factors studied) is more common in diabetics with CKD, this study adds to current knowledge.

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ORIGINAL ARTICLE

Retrospective Study

# Metformin associated lactic acidosis in Auckland City Hospital 2005 to 2009

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Author contributions: Haloob I conceived the study, performed data collection and wrote the initial manuscript while working at Auckland Hospital; de Zoysa JR helped design the study, reviewed data collection and reviewed and revised the manuscript while working at Auckland Hospital.

Institutional review board statement: This retrospective review was approved by the Northern X Regional Ethics Committee (NTX/EXP).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous, de-identified clinical data.

Conflict-of-interest statement: There are no conflicts of interest in the publication of this paper.

Data sharing statement: No additional data are available.



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## Abstract

**AIM:** To determine the incidence, clinical characteristics and outcomes of patients with metformin associated lactic acidosis (MALA).

**METHODS:** Auckland City Hospital drains a population of just over 400000 people. All cases presenting with metabolic acidosis between July 2005 and July 2009 were identified using clinical coding. A retrospective case notes review identified patients with MALA. Prescribing data for metformin was obtained from the national pharmaceutical prescribing scheme.

**RESULTS:** There were 42 cases of metabolic lactic acidosis over 1718000 patient years. There were 51000 patient years of metformin prescribed to patients over the study period. There were thirty two cases of lactic acidosis due to sepsis, seven in patients treated with metformin. Ten cases of MALA were identified. The incidence of MALA was estimated at 19.46 per 100000 patient year exposure to metformin. The relative risk of lactic acidosis in patients on metformin was 13.53 (95%CI: 7.88-21.66) compared to the general population. The mean age of patients with MALA was 63 years, range 40-83 years. A baseline estimated glomerular filtration rate was obtained in all patients and ranged from 23-130 mL/min per 1.73 $m^2$. Only two patients had chronic kidney disease G4.

Three patients required treatment with haemodialysis. Two patients died.

**CONCLUSION:** Lactic acidosis is an uncommon but significant complication of use of metformin which carries a high risk of morbidity.

**Key words:** Acute kidney injury; Lactic acidosis; Metformin



**Core tip:** Metformin is an effective therapy for type 2 diabetes mellitus. Although few side effects are described in clinical trials, here, we describe observational evidence that suggests that use of metformin is associated with an increased risk of lactic acidosis. We recommend dose reduction in the elderly, withholding the drug if an intercurrent illness occurs and that metformin be halted in patients with chronic kidney disease G4.

Haloob I, de Zoysa JR. Metformin associated lactic acidosis in Auckland City Hospital 2005 to 2009. *World J Nephrol* 2016; 5(4): 367-371 Available from: URL: http://www.wjgnet.com/2220-6124/full/v5/i4/367.htm DOI: http://dx.doi.org/10.5527/wjn.v5.i4.367

## INTRODUCTION

The oral hypoglycaemic agent metformin has been used for close to 50 years[1]. It has been found to reduce mortality compared to other agents and is recommended as first line therapy for patients with type 2 diabetes mellitus[2-4]. It is also used for patients with the metabolic syndrome[5] and overweight women with polycystic ovarian syndrome[6].

The biguanide, phenformin, clearly caused lactic acidosis[7]. It has been hypothesized that this severe and significant side-effect is also associated with metformin. Several mechanisms of action have been proposed for the hypoglycaemic effect of the metformin: A reduction in hepatic glucose production, an increase in peripheral glucose uptake, a reduction in gastrointestinal glucose production and a reduction in lipolysis by adipocytes[8]. The major mechanism is through reduction in hepatic production, mediated by phosphorylation of the transcriptional co-activator cAMP response element-binding protein thus reducing the expression of genes inducing gluconeogenesis[9].

It is thought that metformin associated lactic acidosis (MALA) may occur through anaerobic stimulation of lactate production by intestinal cells, with impaired elimination of lactate from the liver and contributed to by accumulation of metformin if there is renal failure, overdose or liver failure[8].

New Zealand has a pharmaceuticals scheme with metformin freely available and fully subsidised. The purpose of this report was to review all cases of lactic acidosis in patients on metformin at Auckland City Hospital.

## MATERIALS AND METHODS

Auckland City Hospital is an adult tertiary referral centre which serves a population of just over 400000 people. Using a health information technology system all cases of metabolic acidosis between July 2005 and July 2009 were identified. Acidosis was defined as a pH $\leqslant$ 7.35. Lactic acidosis was defined as a lactate of $\geqslant$ 5 mmol/L, in association with a low bicarbonate and a low $PCO_2$. Patients with a mixed respiratory and metabolic acidosis were excluded.

The clinical records were available and reviewed for all potential cases. The dose and duration of metformin, other medications, co-morbidities and baseline laboratory data were obtained from the clinical records and primary practice.

Population estimates were obtained from Statistics New Zealand[10]. Data about metformin use in the Auckland region was obtained from the Pharmaceutical Management Agency of New Zealand (PHARMAC). The incidence of diabetes mellitus was estimated from data from the New Zealand Health Survey[11].

Poisson regression statistics were used to determine the risk of lactic acidosis, using the general population as the reference.

## RESULTS

Eight hundred cases of lactic acidosis were identified by the health information technology system. Two hundred and eighty-eight cases of metabolic acidosis were identified by review of laboratory data. Forty-three cases of metabolic lactic acidosis were identified. One was in a nineteen-year-old female, who had an intentional overdose with ten grams of metformin, and was not included in the analysis, thus leaving forty-two cases. Thirty-two patients had metabolic lactic acidosis which was clearly associated with sepsis (Figure 1). Seven of these cases were in patients also taking metformin (Table 1). Four were in patients with diabetes mellitus, not on metformin. Ten patients were taking metformin and did not have a strong alternate cause for lactic acidosis (Table 2).

The population in Auckland City over this period was estimated at 419000 people and increased to 444000 people over the study period. Eighteen point eight percent of the Auckland population are children and cared for by the regional paediatric institution. Thus, we estimate a total of 1395000 patient years over the study period.

The number of patients receiving metformin between July 2005 and July 2009, in Auckland City, was estimated at 51400 patient years. It was estimated that there were 15600 adult patients with diabetes in Auckland each year.

The incidence of metabolic lactic acidosis was

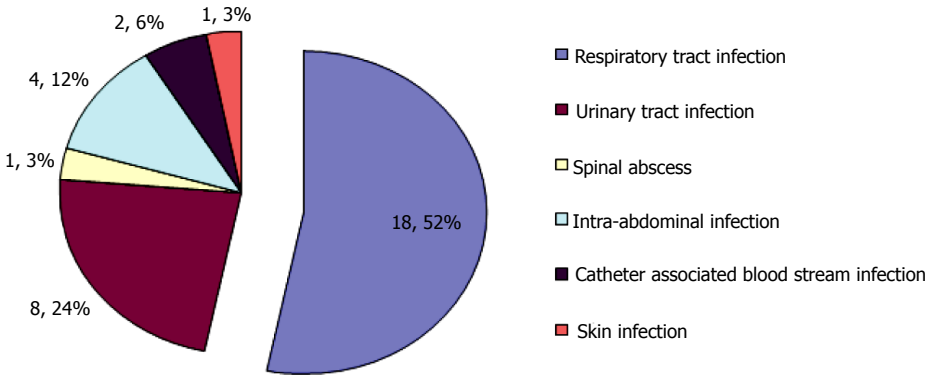


**Figure 1 Cause of sepsis in patients with lactic acidosis.**

**Table 1 Patients on presenting with lactic acidosis and sepsis**

| | All patients | Patients on metformin |
|---|---|---|
| No. of patients | 32 | 7 |
| Age (yr)[1] | 22-85 | 46-81 |
| Sex | 19 females | 4 female |
| Ethnicity | 19 Europeans, 9 Pacific People, 1 NZ Maori, 1 Indian 2 Chinese | 3 Europeans, 3 Pacific People |
| Baseline Creatinine (μmol/L) | 41-200 | 58-140 |
| [2]eGFR mL/min per 1.73 $m^2$ | 25-90 | 31-87 |
| Creatinine at presentation (μmol/L) | 50-600 | 103-463 |
| Number who died | 15 | 3 |
| Number receiving acute haemodialysis | Three | Nil |
| Creatinine at discharge (μmol/L) | 53-245 | 56-60 |
| eGFR mL Tab/min per 1.73 $m^2$ | 22-90 | 77-90 |

[1]The age of the patient is rounded down to the nearest year; [2]eGFR: The estimated glomerular filtration rate based on the four variable MDRD formula[11]. eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

estimated to be 3.01 per 100000 patient years for the general population. The incidence of metabolic lactic acidosis due to sepsis was estimated as 2.29 per 100000 patient years.

The incidence of metabolic lactic acidosis was 33.07 per 100000 patient years' exposure to metformin. There was a significant increase in the relative risk of lactic acidosis in patients on metformin compared to the general population RR = 13.53 (95%CI: 7.88-21.66).

The incidence of MALA was 19.46 per 100000 patient years exposure to metformin. There were four male and six female patients whose mean age was 63 years, range 40-83 years (Table 2). All patients were prescribed metformin for type 2 diabetes mellitus and were also on either an angiotensin converting enzyme inhibitors or an angiotensin two receptor antagonist. Four patients presented with congestive heart failure, two patients had ischaemic events, three patients had gastroenteritis and one patient had a bradyarrhythmia as the primary diagnosis. All patients had their renal function tested in the community prior to their presenting illness; the baseline eGFR, as determined using the modified MDRD formula[12], ranged from 23-90 mL/min per 1.73 $m^2$. Only two patients had chronic kidney disease (CKD) 4, five patients had CKD3. In addition to other therapy three patients were treated with emergent haemodialysis (patients 1, 3 and 9, Table 2). Two patients died, one of cardiac ischaemia and one of multi-organ failure (patient 1 and 5 respectively, Table 2).

## DISCUSSION

Metformin remains an attractive option in the treatment of type 2 diabetes: It promotes weight loss and has been shown to reduce the complications of and the mortality associated with diabetes[13]. Monotherapy with metformin appears to carry greater benefits than monotherapy with other hypoglycaemic agents[14].

The main concern with the use of metformin is the risk of developing lactic acidosis. Although a number of case series exist in the literature it is controversial as to whether MALA actually occurs. In a Cochrane review, with 70490 patient years of exposure to metformin, no cases of MALA were identified[14]. It was estimated that the hypothetical incidence of lactic acidosis in patients treated with metformin was 4.3 per 100000 patient years and 5.4 per 100000 patient years in non-metformin users[15]. In our series we report a low rate of lactic acidosis in the general population but that the rate of lactic acidosis in patients on metformin is significantly greater. Other population based studies have also reported a much greater rate of lactic acidosis in patients on metformin: In a recent series Scale and Harvey reported a rate of 120 per 100000 patient years[16]. There are several potential reasons for the discrepancy between the observational studies and the clinical trial cohorts. Lactic acidosis is uncommon and may occur some time after the initiation of therapy, and thus may be missed in studies with short term follow-up. Lactic acidosis is not commonly listed as a primary discharge diagnosis, and thus may be underdiagnosed. There may be reporting bias in clinical trials. In addition, clinical trials may exclude patients such

**Table 2 Demographic and clinical details of patients with metformin associated lactic acidosis**

| Patients | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Age (yr)[1] | 68 | 53 | 68 | 63 | 80 | 40 | 83 | 55 | 67 | 72 |
| Sex | Male | Male | Male | Female | Male | Female | Female | Female | Female | Female |
| Ethnicity | Pacific Islander | Indian | European | Pacific Islander | Pacific Islander | Pacific Islander | Pacific Islander | Pacific Islander | European | European |
| Metformin dose (g/d) | 2.5 | 1.7 | 2 | 3 | 2 | 2 | 1 | 2 | 1.7 | 1 |
| Duration of Metformin[2] | 5 yr | 4 yr | 5 yr | 4 yr | 2 yr | 1 yr | 4 yr | 4 yr | 4 yr | 2 mo |
| Baseline Creatinine (μmol/L) | 114 | 57 | 138 | 123 | 106 | 123 | 180 | 105 | 154 | 139 |
| [3]eGFR mL/min per 1.73 $m^2$ | 55 | 130 | 44 | 38 | 58 | 42 | 23 | 47 | 29 | 32 |
| Creatinine at Presentation (μmol/L) | 449 | 90 | 333 | 612 | 381 | 612 | 376 | 895 | 973 | 304 |
| pH | 7.3 | 7.23 | 7.14 | 7.32 | 6.99 | 7.21 | 7.34 | 6.9 | 7.35 | 7.09 |
| Bicarbonate (mmol/L) | 15 | 13 | 13 | 16 | 6 | 12 | 12 | 3 | 15 | 15 |
| Lactate (mmol/L) | 9.2 | 6.7 | 15 | 6 | 16 | 8.4 | 6 | 22 | 6.2 | 7 |
| Received acute haemodialysis | Yes | No | Yes | No | No | No | No | No | Yes | No |
| Outcome | Dead | Alive | Alive | Alive | Dead | Alive | Alive | Alive | Alive | Alive |
| Creatinine at discharge (μmol/L) | - | 73 | 121 | 95 | - | 90 | 167 | 123 | 129 | 96 |
| [3]eGFR mL/min per 1.73 $m^2$ | - | 97 | 52 | 52 | - | 60 | 25 | 39 | 36 | 50 |

[1]The age of the patient is rounded down to the nearest year; [2]The duration of metformin is rounded down to completed years of therapy, except for patient 10; [3]eGFR: The estimated glomerular filtration rate based on the four variable MDRD formula[11]. eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

as the elderly or other with co-morbidities that may also contribute to the risk of developing lactic acidosis.

We used data from the New Zealand health survey to estimate the incidence of diabetes in Auckland. This survey estimated the prevalence of diabetes in children as 0.1%-0.4% and the number of diagnosed adult patients at between 3.4% to 6.3%. This is in line with national estimates but does not account potential patients with undiagnosed diabetes. Thus, we are likely to be underestimating the overall incidence of diabetes in the region. We used data from Pharmac to estimate the use of metformin in the region. Pharmac records the subsided use of metformin. Currently, all New Zealanders enrolled with a general practice are eligible for subsidised metformin and a free health check if they have diabetes. However, this does not extend to non-New Zealand residents in the region. Further, if a number of patients with diabetes are not enrolled with a primary practice, then they are also ineligible for subsidised metformin. Finally, a phased rollout of subsidized medications occurred between 2003 and 2007. All of these factors may also lead to underestimation of the use of metformin in the region.

We describe a series of ten patients with MALA. The mortality in this group of patients is high but not as great as that seen in lactic acidosis associated with sepsis, and all cases were associated with acute kidney injury. Renal replacement therapy is an attractive therapeutic option as it aids in the correction of acidosis and also the removal of lactate and metformin. However, it is not clear that haemodialysis confers any survival benefit[17]. In our series only three patients received dialysis. Interestingly, the patient who presented with the worst laboratory parameters, case 8, was managed with supportive therapy, did not receive dialysis, and survived with recovery of her renal function.

Clearly metformin is an effective therapy. Here we describe observational evidence that suggests that use of metformin is associated with an increased risk of lactic acidosis. The standard recommendations are to use metformin cautiously in patients with hepatic impairment and reduce the dose in the elderly. We recommend reducing the dose of metformin in CKD G4 and advise stopping when the eGFR is less than 20 mL/min. We suspect that this later message is well heeded, and may be a reason that only two patients in our series with CKD G4 were found to develop MALA. In addition, we routinely recommend to patients that if they develop an intercurrent illness that metformin be withheld and medical review is sought.

Further investigation of this issue is suggested to confirm the findings in this study, using more robust design and controlling more potential confounding factors, *e.g.,* indication for metformin, co-morbidities and age.

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## COMMENTS

### *Background*

The incidence of metformin associated lactic acidosis (MALA) is small when assessed by systematic review, however, randomised controlled trials may underestimate the true incidence by using strict inclusion and exclusion criteria. This retrospective review describes the incidence of MALA and highlights the significant morbidity and mortality that is associated with this condition.

### *Research frontiers*

No randomised clinical trial has been undertaken to assess the safety of metformin in patients with mild to moderate renal impairment. This would be

challenging due to the low incidence of MALA. Use of observational cohort data or national patient registries may better quantify risk and acceptable clinical practice.

### Applications

The authors recognise the efficacy of metformin as a therapeutic agent for type 2 diabetes and recommend reducing the dose of metformin in mild to moderate renal impairment, and advise halting metformin when the estimated glomerular filtration rate is less than 20 mL/min. In addition, they recommend to patients that if they develop an intercurrent illness that metformin be withheld and medical review sought.

### Peer-review

The paper gives interesting information about the incidence of metformin associated lactic acidosis which is important for clinical practice. It is well written and the analysis has been performed adequately.

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ORIGINAL ARTICLE

Observational Study

# Skin disorders in peritoneal dialysis patients: An underdiagnosed subject

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## Abstract

**AIM:** To examine all skin changes in peritoneal dialysis (PD) patients followed up in our unit.

**METHODS:** Patients on PD program for at least three months without any known chronic skin disease were included in the study. Patients with already diagnosed skin disease, those who have systemic diseases that may cause skin lesions, patients with malignancies and those who did not give informed consent were excluded from the study. All patients were examined by the same predetermined dermatologist with all findings recorded. The demographic, clinical and laboratory data including measures of dialysis adequacy of patients were recorded also. Statistical Package for Social Sciences (SPSS) for Windows 16.0 standard version was used for statistical analysis.

**RESULTS:** Among the patients followed up in our PD unit, those without exclusion criteria who gave informed consent, 38 patients were included in the study with male/female ratio and mean age of 26/12 and 50.3 ± 13.7 years, respectively. The duration of CKD was 7.86 ± 4.16 years and the mean PD duration was 47.1 ± 29.6 mo. Primary kidney disease was diabetic nephropathy in 11, nephrosclerosis in six, uropathologies in four, chronic glomerulonephritis in three, chronic pyelonephritis in three, autosomal dominant polycystic kidney disease in three patients while cause was unknown in eight patients. All patients except for one patient had at least one skin lesion. Loss of lunula, onychomycosis and tinea pedis are the most frequent skin disorders recorded in the study group. Diabetic patients had tinea pedis more

frequently ($P$ = 0.045). No relationship of skin findings was detected with primary renal diseases, comorbidities and medications that the patients were using.

**CONCLUSION:** Skin abnormalities are common in in PD patients. The most frequent skin pathologies are onychomycosis and tinea pedis which must not be overlooked.

**Key words:** Skin; Peritoneal dialysis; Onychomycosis; Tinea pedis; Xeroderma



**Core tip:** Skin abnormalities are common in peritoneal dialysis patients. We aimed in our study to examine all skin changes in peritoneal dialysis patients followed up in our unit. Among the 38 patients included, all but one patient had at least one skin lesion. Loss of lunula, onychomycosis and tinea pedis are the most frequent skin disorders recorded in the study group. Diabetic patients had tinea pedis more frequently. No relationship of skin findings was detected with primary renal diseases, comorbidities and medications that the patients were using. Skin changes are commonly overlooked and should be sought for timely diagnosis and treatment.

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## INTRODUCTION

The chronic uremic status and the concomitant metabolic disorders may lead to a variety of structural and functional changes in the skin and its appendages. Dermatologic abnormalities are common in chronic kidney disease (CKD) and almost all patients have at least one of the cutaneous involvements[1]. These abnormalities range from the frequently seen xerosis and pruritus to the more rare disorders like hyperpigmentation, purpuric skin changes, acquired perforating dermatosis, and nail abnormalities[1-3]. Some of these disorders are described specifically in end stage renal disease (ESRD) like acquired perforating dermatosis, bullous dermatoses, metastatic calcification, and nephrogenic systemic fibrosis, while the others are nonspecific findings that may be associated with various entities. These include pruritus, color changes, xerosis, and half-and-half nails[4]. Symptoms associated with skin disorders can lead to varying degrees of discomfort, anxiety, depression, sleeping disorders and can affect the quality of life leading to distorted mental and physical health[5].

The skin changes observed in hemodialysis (HD) patients have been studied previously[2,6,7]. But, data about peritoneal dialysis (PD) patients regarding skin changes is lacking in the literature except for a few studies subjecting only skin color changes and xerosis[8-10] and another study reported in 1992[1].

We aimed in our study to examine all skin changes in PD patients followed up in our PD unit.

## MATERIALS AND METHODS

Patients who gave informed consent among those who were on PD program for at least three months and followed up in our PD unit were included in the study. Patients were using either conventional glucose based solutions or biocompatible solutions as well as icodextrin. Patients with already diagnosed skin disease, those who have systemic diseases that may cause skin lesions, patients with malignancies and those who did not give informed consent were excluded from the study. All patients were examined once by the same predetermined dermatologist with all findings recorded. Baseline data including age, gender, concomitant diseases, duration of CKD and PD therapy, and the medications used by each patient were recorded. Concurrent medications and dose of monthly erythropoietin were also documented. Laboratory investigations in the form of complete blood counts, blood glucose, urea, creatinin, uric acid, aspartate transaminase, alanine transaminase, alkaline phosphatase, gamma glutamyl transferase, total protein, albumin, bilirubin, electrolytes, calcium, phosphorus, parathyroid hormone, ferritin, transferrin saturation, vitamin B12, folic acid, total cholesterol, Low-density lipoprotein cholesterol, and triglyceride levels at the time of physical examination and hepatitis panel collected from the most recent data in the patients' files were recorded. Among PD related parameters, weekly Kt/V urea, peritoneal Kt/V urea, residual renal glomerular filtration rate (GFR) and the transport type of the patients were obtained.

### *Statistical analysis*

Statistical Package for Social Sciences (SPSS) for Windows 16.0 standard version was used for statistical analysis. Numerical parameters were expressed as mean ± SD. Intergroup comparisons of nonnumeric parameters were done by $\chi^2$ test were used. $P$ values less than 0.05 were accepted as statistically significant.

## RESULTS

Among the 52 patients followed up in our PD unit, three patients were already on treatment for a symptomatic skin disorder (one for psoriasis, two for xerosis cutis), one patient had breast cancer and 10 patients rejected to be examined by the dermatologist. The remaining 38 patients were included in the study. Female/male ratio and the mean age were 26/12 and 50.3 ± 13.7 years, respectively. The duration of CKD was 94.3 ± 49.9 mo and the mean PD duration was 47.1 ± 29.6 mo. The PD modality was continuous ambulatory peritoneal dialysis

**Table 1 Biochemical and hematological laboratory data of the patients**

| Parameter | Mean ± SD | Parameter | Mean ± SD |
|---|---|---|---|
| Hemoglobin (g/dL) | 10.9 ± 1.3 | Phosphorus (mg/dL) | 4.7 ± 1.0 |
| Hematocrit (%) | 32 ± 3 | Parathyroid hormone (pg/mL) | 600 ± 502 |
| Ferritin (ng/dL) | 288 ± 294 | Alkaline phosphatase (U/L) | 180 ± 342 |
| Transferrin saturation (%) | 25 ± 10 | Alanine transaminase (U/L) | 12.8 ± 5.4 |
| Total protein (g/dL) | 6.9 ± 0.5 | Aspartate transaminase (U/L) | 16.2 ± 6.6 |
| Albumin (g/dL) | 3.5 ± 0.3 | Total bilirubin (mg/dL) | 0.5 ± 0.4 |
| Uric acid (mg/dL) | 5.6 ± 1.0 | Direct bilirubin (mg/dL) | 0.1 ± 0.02 |
| Calcium (mg/dL) | 8.9 ± 0.6 | Gamma glutamyl transferase (U/L) | 24.8 ± 22.4 |

**Table 2 The medications used by the patients**

| Drug | *n* (%) | Drug | *n* (%) |
|---|---|---|---|
| Calcium-containing phosphorus binders | 27 (71) | Alpha blockers | 6 (16) |
| Diuretics | 25 (66) | Acetylsalicylic acid | 6 (16) |
| Active vitamin D | 21 (55) | RAS blockers | 5 (13) |
| Erythropoiesis stimulating agents | 20 (53) | Cinecalcet | 5 (13) |
| Calcium channel blockers | 18 (47) | Allopurinol | 3 (7) |
| Beta blockers | 13 (34) | Fibrates | 1 (2.6) |
| Statins | 14 (37) | Sevalemer | 1 (2.6) |
| Essential amino acid | 10 (26) | | |

RAS: Renin-angiootensin-aldosteron system.

(CAPD) in 31 patients and automated peritoneal dialysis (APD) in seven patients. Diabetes mellitus was the most common cause of ESRD ($n$ = 11, 28.9%). Other causes of ESRD were hypertensive nephrosclerosis ($n$ = 6, 15.7%), urological disorders ($n$ = 4, 10.5%), chronic glomerulonephritis ($n$ = 3, 7.8%), chronic pyelonephritis ($n$ = 3, 7.8%), autosomal dominant polycystic kidney disease ($n$ = 3, 7.8%); while the etiology was not known in the remaining eight patients (21%). Hypertension ($n$ = 24, 63.1%), diabetes mellitus ($n$ = 13, 34.2%), hyperlipidemia ($n$ = 11, 28.9%), hypothyroidism ($n$ = 8, 21%), ischemic heart disease ($n$ = 7, 18.4%), malignancies ($n$ = 3, 7.8%), cerebrovascular disease ($n$ = 1, 2.6%) were recorded as comorbidities.

The biochemical and hematological laboratory data of the patients are presented in Table 1. The mean Kt/V urea and weekly creatinine clearance values were 2.46 ± 0.67 and 78 ± 33 L/wk per 1.73 $m^2$, respectively. The medications that the patients were using are presented in Table 2.

All patients except for one patient had at least one skin lesion. The skin disorders recorded in patients are presented in Table 3. Loss of lunula, onychomycosis and tinea pedis are the most frequent skin disorders recorded in the study group.

Diabetic and nondiabetic patients were similar regarding skin findings except for tinea pedis which was more common in diabetic patients ($n$ = 8, 61% *vs* $n$ = 7, 28%; $P$ = 0.045). Patients using erythropoiesis stimulating agents have lower rate of xeroderma cutis compared to those not using them ($n$ = 11, 55% *vs* $n$ = 3, 17%; $P$ = 0.014) as well as lower rate of onychomycosis ($n$ = 5, 25% *vs* $n$ = 11, 61%; $P$ = 0.024). Loss of lunula was more rare in patients on statin treatment ($n$ = 1, 7% *vs* $n$ = 16, 67%; $P$ < 0.001). Patients using diuretics had higher rate of tinea pedis ($n$ = 13, 52% *vs* $n$ = 2, 15%; $P$ = 0.028). No relationship of skin findings was detected with primary renal diseases, comorbidities and medications that the patients were using.

## DISCUSSION

Skin abnormalities are common in patients with ESRD. Previous studies were mostly about the skin findings in patients on HD treatment. On the other hand, studies about dermatological abnormalities in PD patients are limited to a few studies in which only hyperpigmentation and xerosis were searched for, and an old study in which PD patients were regarded as a separate group[1,8-10].

It was reported in the study by Picó *et al*[1] that patients on different dialytic treatments have different skin abnormalities. The pathologies underlying skin changes in uremic patients are accumulation of uremic toxins, metabolic abnormalities and dryness of the skin[11-13]. Besides, there are findings supporting the role of the type of dialysis on the profile of skin changes[2,14]. It has been reported that signs and symptoms related to skin increase after starting HD treatment[2]. There may also be role of the apparatus used during dialysis and chemical irritation due to dialysis solutions besides dialysis adequacy. In fact, allergic skin reactions have been reported in 10% of patients using icodextrin[15].

We evaluated in our study the prevalence of skin abnormalities in patients on PD treatment and its relationship with primary renal disorder, comorbidities and the medications.

The most frequent skin finding in our study population was loss of lunula which was observed in 44.7% of our patients. No data was found in the literature about loss

**Table 3 The skin findings of the patients**

| Lesion | *n* (%) | Lesion | *n* (%) |
|---|---|---|---|
| Loss of lunula | 17 (44.7) | Koilonychia | 1 (2.6) |
| Onychomycosis | 16 (42.1) | Pigmented purpuric dermatosis | 1 (2.6) |
| Tinea pedis | 15 (39.5) | Neurodermitis | 1 (2.6) |
| Xeroderma cutis | 14 (36.8) | Prurigo nodularis | 1 (2.6) |
| Hyperpigmentation | 11 (28.9) | Splinter hemorrhage | 1 (2.6) |
| Nevus | 6 (15.8) | Subungual hyperkeratosis | 1 (2.6) |
| Acne | 4 (10.5) | Verruca vulgaris | 1 (2.6) |
| Uremic pruritus | 3 (8.1) | Vitiligo | 1 (2.6) |
| Contact dermatitis | 2 (5.3) | Half and half nail | 1 (2.6) |
| Folliculitis | 2 (5.3) | Acne rosacea | 1 (2.6) |
| Chronic eczema | 1 (2.6) | | |

of lunula in PD patients. Ozturk *et al*[16] reported in their study related to nail changes in HD patients, that loss of lunula was present in 58% of HD patients while the rate was 8% in the control group. Renal transplant recipients were compared with healthy subjects regarding nail changes in Egypt[17]. The rates were similar in both groups (30% *vs* 26%), and the finding was accepted as a normal variation[17].

Half and half nail was detected in only one patient in our study, while it was reported at an average rate of 20% in studies reaching even 76%[4,18,19]. Ozturk *et al*[16] reported that half and half nail was present in 15% of the HD patients involved in their study. Picó *et al*[1] also reported increased frequency of this abnormality in HD patients. All these findings lead to the idea that half and half nail may be related with HD specifically.

Hyperpigmentation was observed 28.9% of patients in our study. Increased melanocyte stimulating hormone levels, increased dermal melanin density, dermal accumulation of urochrome pigments and carotenoids are responsible for hyperpigmentation in patients with ESRD[14,20]. Increased length of time on dialysis and loss of residual renal functions increase the frequency of hyperpigmentation. Hyperpigmentation has been reported to be present in patients with ESRD at rates between 17% and 22%[1,2,21]. The frequency of splinter hemorrhages and echymoses was higher in relatively old studies, while their rates have decreased in recent studies[14]. Patients in our study did not have any sign of skin hemorrhage.

Xerosis cutis is one of the most frequent skin lesions in patients with ESRD. Besides decreasing the quality of life, xerosis caused delayed wound healing and propensity to skin infections[13,22]. The rate of xerosis cutis in the literature is about 50%-85% while the corresponding number in our study is 36.8%[23]. Morton *et al*[10] found higher incidence of xerosis and pruritus in PD patients compared to HD patients. They stated that this difference may be related to defects in calcium homeostasis.

We detected onychomycosis and tinea pedis in 42.1% and 39.5% of the patients, respectively in our study. The corresponding rates were 52% and 25% in the study by Picó *et al*[1] which is the single study in which skin findings of PD patients were evaluated. Moreover, the authors stated that they were more frequent in diabetic PD patients compared to HD patients and non-diabetic counterparts respectively. Tinea pedis was more frequent in diabetic subjects in our study also ($P$ = 0.045). The glucose content of dialysis solutions and the resultant worsening in glucose regulation may cause a propensity for infection.

Patients using diuretics had higher rate of tinea pedis ($P$ = 0.028). This may be related with hypervolemia and so edema which necessitates use of diuretics. But it can be just a speculation, because clinical findings of patients were not recorded.

There was no correlation between the frequency of skin lesions and other comorbid diseases, dialysis adequacy parameters and metabolic abnormalities including hyperphosphatemia.

Patients using erythropoiesis stimulating agents have lower rate of xeroderma cutis and lower rate of onychomycosis compared to those not using them.

The other less frequent skin findings detected in our patients are presented in Table 2. It was striking that acquired perforating dermatosis, bullous dermatoses, metastatic calcification and calciphylaxis which are regarded as specific manifestations of HD patients and related to mortality in some cases, were not reported in our study group[14,24,25]. There was no control group consisting of HD in our study; but when compared with the results of studies carried out with HD patients, PD patients seem to be protected from severe skin lesions. Onychomycosis and tinea pedis comprised the majority of skin pathologies in our study[1].

There may be several reasons for the difference between these two dialysis modalities regarding patterns of skin pathologies. The ultrafiltration process spread to 24 h protects PD patients from hemodynamic instability; and generally prevents excessive ultrafiltration. So, a more stable hemodynamic status provides better and continuous tissue perfusion. More importantly, HD procedure itself may cause skin hypoxia. Previous studies showed that transdermal oxygen perfusion may decrease by as much as 15-20 mmHg during hemodialysis and dermal microcirculation may be distorted[26,27]. The type of the HD membrane used may be effective on this effect[28]. The involvement of skin, which is the most distal organ in

the body, is a predictable result of hypoxemia and tissue hypoxia. PD, with more stable hemodynamic status and better skin oxygenation, may allow lesions easy to cope with to be more frequent. On the other hand, exposure to high amount of glucose for a prolonged time may increase the frequency of fungal skin infections in both diabetic and nondiabetic PD patients.

Skin abnormalities are common in in PD patients. The spectrum of clinical presentation is different from HD patients based on recent reports. The most frequent skin pathologies are onychomycosis and tinea pedis which must not be overlooked.

## COMMENTS

### Background

Dermatologic abnormalities are common in chronic kidney disease and almost all patients have at least one of the cutaneous involvements. Some of these disorders are described specifically in end stage renal disease while the others are nonspecific findings that may be associated with various entities. Symptoms associated with skin disorders can lead to varying degrees of discomfort, anxiety, depression, sleeping disorders and can affect the quality of life leading to distorted mental and physical health.

### Research frontiers

The skin changes observed in hemodialysis patients have been studied previously. But, data about peritoneal dialysis patients regarding skin changes is lacking in the literature except for a few studies subjecting only skin color changes and xerosis. The authors aimed to examine all skin changes in peritoneal dialysis (PD) patients followed up in the authors' PD unit.

### Innovations and breakthroughs

Data about peritoneal dialysis patients regarding skin changes is limited in the literature. This study showed that skin abnormalities are common in PD patients. The spectrum of clinical presentation is different from hemodialysis patients based on recent reports. The most frequent skin pathologies are onychomycosis and tinea pedis which must not be overlooked.

### Terminology

Peritoneal dialysis is an option for patients with end stage renal disease. The most commonly encountered skin lesion in chronic kidney disease are xerosis and pruritus. Xerosis is abnormal dryness of the skin which also may cause pruritus.

### Peer-review

Skin abnormalities are common in in PD patients. The most frequent skin pathologies are onychomycosis and tinea pedis which must not be overlooked.

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# Measurement of the intestinal permeability in chronic kidney disease

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Author contributions: Terpstra ML and Singh R performed the electronic search, all co-authors searched their own personal databases; Terpstra ML and Singh R independently screened all the articles for meeting the inclusion criteria; Bemelman FJ was consulted if there was discussion about inclusion; Terpstra ML extracted all data and wrote the paper under supervision of Geerlings SE and Bemelman FJ.

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## Abstract

**AIM:** To evaluate methods measuring the intestinal permeability in chronic kidney disease (CKD) and clarify whether there is an increased intestinal permeability in CKD.

**METHODS:** We reviewed the literature in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) protocol and performed a systematic literature search through MEDline and EMBASE. All controlled trials and cohort studies using non-invasive methods to assess intestinal permeability in CKD patients were included. Excluded were: Conference abstracts and studies including patients younger than 18 years or animals. From the included studies we summarized the used methods and their advantages and disadvantages. For the comparison of their results we divided the included studies in two categories based on their included patient population, either assessing the intestinal permeability in mild to moderate CKD patients or in end stage renal disease (ESRD) patients. Results were graphically displayed in two plots, one comparing the intestinal permeability in mild to moderate CKD patients to healthy controls and one comparing the intestinal permeability in ESRD patients to healthy controls.

**RESULTS:** From the 480 identified reports, 15 met our inclusion criteria. Methods that were used to assess the intestinal permeability varied from markers measured in plasma to methods based on calculating the urinary excretion of an orally administered test substance. None of the applied methods has been validated in CKD patients and the influence of decreased renal function on the different methods remains unclear to a certain extent. Methods that seem the least likely to be influenced by decreased renal function are the quantitative PCR (qPCR) for bacterial DNA in blood and D-lactate. Considering

the results published by the included studies; the studies including patients with mild to moderate CKD conducted conflicting results. Some studies did report an increase in intestinal permeability whilst other did not find a significant increased permeability. However, despite the variety in used methods among the different studies, all studies measuring the intestinal permeability in ESRD point out a significant increased intestinal permeability. Results should nevertheless be interpreted with caution due to the possible influence of a decreased glomerular filtration rate on test results.

**CONCLUSION:** The intestinal permeability in CKD: (1) could be measured by qPCR for bacterial DNA in blood and D-lactate; and (2) seems to be increased in ESRD.

**Key words:** Chronic kidney disease; Intestinal barrier function; Intestinal permeability; Markers; Renal failure



**Core tip:** Several methods are currently being used to measure the intestinal permeability, there is however no gold standard. In addition to this, most methods are influenced by renal function. We suggest that preferred methods to assess the intestinal permeability in chronic kidney disease patients could be quantitative PCR for bacterial DNA in blood and D-lactate. Independent of the used method, all studies measuring the intestinal permeability in patients with end stage renal disease (ESRD) reported a significantly increased intestinal permeability. Even though these results should be interpret with caution due to the disadvantages of the applied methods, it seems likely that there is a connection between ESRD and intestinal barrier dysfunction.

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## INTRODUCTION

Within the last three decades an increasing number of studies highlight the role of chronic systemic inflammation in the progression of chronic kidney disease (CKD) to end stage renal disease (ESRD) and its associated complications, such as cardiovascular disease[1,2]. Even though the inflammatory status has been pointed out as an important prognostic factor in CKD, the pathophysiology has not been elucidated. Factors that appear to be involved are retained uremic toxins, hypervolemia, hypertension, underlying disease (diabetes, autoimmune disease, *etc.*) and infection[3,4]. In addition to this, more recent studies have been opposing alterations in the gut as possible source of inflammation[5-7].

An important aspect of the alterations in the gut is a decreased barrier function causing an increased intestinal permeability, which possibly leads to diffusion of endotoxins and bacterial DNA through the epithelial barrier into the circulation. An interesting finding was reported in uremic rats; gut bacteria and their DNA fragments were found in the intestinal wall and the mesenteric lymph nodes, whilst their non-uremic controls showed no signs of these fragments in the obtained biopsies[8]. The entry of uremic retention solutes, bacterial DNA, endotoxins and other possibly noxious compounds from the intestinal lumen into the circulation is likely to contribute to the inflammatory status of CKD patients and thus their prognosis.

The suggestion of an increased intestinal permeability as a prognostic factor in CKD has led to an increased interest in non-invasive methods measuring the intestinal permeability in CKD patients. There are numerous approved ways to assess the intestinal permeability, which was outlined in 2010 by Grootjans *et al*[9]. There is however no gold standard and each method comes with its own advantages and disadvantages. An important aspect is how renal function interferes with the test results; most studies assessing the intestinal permeability exclude CKD patients to prevent possible bias obtained by a decreased estimated glomerular filtration rate (eGFR).

Presently there is no overview available on the results of studies assessing the intestinal permeability in CKD.

This systematic review provides an overview of the studies assessing the intestinal permeability of the small and large intestine in CKD patients. We will answer two research questions: (1) what is the best available method to determine the intestinal permeability in CKD patients; and (2) what is currently known on intestinal permeability in CKD.

We discuss the methods used to assess the intestinal permeability in CKD patients and their advantages and disadvantages, specifically focusing on the influence of renal function. In addition to this we extracted the data derived from these studies in order to summarize the results of the currently available evidence of what is known about the intestinal permeability in CKD.

## MATERIALS AND METHODS

We reviewed the literature in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol[10]. The study protocol was registered in the PROSPERO international registry; registration number CRD42015025101. PROSPERO is an international database of prospectively registered reviews in health and social care in which key features from the review protocol are recorded and maintained as a permanent record. PROSPERO aims to provide a comprehensive listing of systematic reviews registered at inception to help avoid unplanned duplication and enable comparison of reported review methods with what was planned in the protocol[11].

Two co-authors (Terpstra ML and Singh R) performed

a systematic literature search through MEDLINE and EMBASE, combined with a search through personal databases of all co-authors. Search terms for each database are described in supplementary tables.

The references obtained through the search were stored within Endnote X7 file. Titles and abstracts of the obtained articles were screened by two co-authors independently, Terpstra ML and Singh R. In case of discussion about inclusion, a third investigator was consulted (Bemelman FJ). All trials and cohort studies using non-invasive methods to assess the permeability of the small and large intestine in CKD patients were included. Only methods directly reflecting the intestinal permeability of a patient at a specific time were included, studies demonstrating the effect of different compounds on the intestinal barrier were excluded. Furthermore studies only assessing the permeability of the stomach were excluded since there are no uremic and other noxious compounds produced here and the environment is almost sterile.

Other exclusion criteria were: Conference abstracts, patients younger than 18 years and animal studies. Studies reporting data that had been previously published were also excluded.

Whilst analyzing the publications possibly meeting the inclusion criteria; articles cited in the included studies were also assessed on their relevance and included when meeting the eligibility criteria.

Each reference was categorized in Endnote according to the inclusion/exclusion criteria.

For each included study the methodological quality assessment was provided by using the Newcastle - Ottowa quality assessment scale for cohort studies[12]. For this scorings system not all items were applicable to the type of included studies; points were only given for those sections that were relevant. Hence, the maximum amount of stars that could be obtained was 6.

From the included studies the following data were retrieved: Data on CKD etiology and renal function, sample size of the subgroups, description of control group, method(s) used to assess the permeability, part of the intestine that is evaluated by this method, mean or median levels of the used marker per subgroup (if provided) and *P* value of the statistical test that was used to compare the groups. If applicable, the interaction between the measurement outcome and renal function was evaluated; whether or not the measurement outcome was corrected for the renal function.

All data were summarized in two tables: Table 1 summarizes the mechanism of action and (dis)advantages of each method and Table 2 summarizes the results obtained by each study.

In attempt to compare results studies were divided in two categories: Studies comparing the intestinal permeability in mild to moderate CKD patients (eGFR 15-90) to healthy controls and studies comparing intestinal permeability in ESRD [eGFR < 15; both hemodialysis (HD) and non-hemodialysis (non-HD)] patients to healthy controls. For each study providing the mean and standard deviation the standardized mean difference was calculated through Review Manager 5.3. Biostatistics analysis was performed after consultation of a biomedical epidemiologist. In case of missing data the authors of the studies were contacted in order to obtain the required data. If studies only provided mean and standard deviation values of subgroups, we calculated the mean and standard deviation for the entire group with the following formula: sqrt[(6-1)*1.47^2+(24-1)*2.28^2]/(6-1+24-1). $Sp = \sqrt{(n1 - 1) \times S1^2 + (N2 - 1) \times S2^2 / (n1 - 1 + n2 - 1)}$[13].

Results were graphically displayed in two plots, one comparing the intestinal permeability in mild to moderate CKD patients to healthy controls and one comparing the intestinal permeability in ESRD patients to healthy controls. Since different methods were used among the different included studies, results were not pooled and no meta-analysis was performed.

## RESULTS

Our search through MEDLINE and EMBASE yielded 646 articles. The personal databases retrieved one more article and the search through the references lists of the relevant studies yielded three more studies. After removing duplicates, 480 articles remained and were screened for meeting the inclusion criteria. In 24 articles the full text was assessed. Reasons for exclusion are summarized in Figure 1. A total number of 15 studies were included in our study.

For each included study the methodological study was assessed through the Newscastle - Ottowa quality assessment scale[12]. The amount of stars scored by each study is summarized in supplementary tables. The mean amount of stars obtained by each study was 4.7 with a range from 4 to 6 stars. Methods that were used to assess the intestinal permeability varied from markers measured in plasma to methods based on calculating the urinary excretion of an orally administered test substance. The used methods, their mechanisms of action and (dis)advantages are summarized in Table 1. Most commonly used were the sugar absorption test[14-17], D-lactate (plasma)[18,19] and chromium-51 labeled ethylenediamine tetra acetic acid ($^{51}$Cr-EDTA) (plasma)[20-23]. More recent studies focused on bacterial DNA[18,19,24] and endotoxins or LPS[16,18,25-27] in blood as a projection of intestinal permeability. Few studies used other methods such as polyethylene glycols (PEGs) in urine[28].

Results provided by each included study are summarized in Table 2. From the 15 included studies, 7 studies provided sufficient data to calculate the mean differences: 4 studies comparing the mild to moderate CKD patients to healthy controls and 3 studies comparing the ESRD patients to the healthy control population (Figures 2 and 3).

Despite the variety in used methods among the different studies, results considering the ESRD patient population are uniform. As displayed in Figure 3, all studies comparing ESRD patients to healthy controls point out a significantly increased intestinal permeability,

**Table 1 Characteristics of the used methods assessing the intestinal permeability**

| Marker for intestinal permeability | Mechanism of action | Advantages | Disadvantages | Influence renal function | Part of the intestine evaluated | Ref. |
|---|---|---|---|---|---|---|
| D-lactate (plasma) | Produced by bacteria in the colon. Present in human blood at very low concentrations as a product of methylglyoxal metabolism. In case of increased intestinal permeability levels will rise due to increased translocation across the intestinal mucosa | Non-invasive<br>Low levels in healthy subjects, high specificity<br>Mainly large intestine; thus focusing on part of the bowel with the highest bacterial load | Possibly increased fermentation of undigested carbohydrates to D-lactate in case of bacterial overgrowth | Influenced by renal function to some extent | Mainly large intestine | [18,19] |
| Sugar absorption test (urine) | Method based on calculating the urinary excretion of orally administered test substance that reflects the non-mediated diffusion of that probe across the intestinal barrier. Most commonly used combination of sugars is a oligosaccharide or disaccharide (lactulose, cellobiose) combined with a monosaccharide (mannitol). By adding sucralose to the test, which is not degraded by the bacteria of the colon, the colonic permeability can be assessed | Non-invasive<br>Different sugar combinations can assess different parts of the gastrointestinal tract | Relative impractical in use<br>Results could be influenced by decreased bowel motility 32<br>Used according to different protocols and different combinations of sugars which makes the comparison of studies difficult<br>Relative large inter- and intra-individual variety | Influenced by renal function. Corrected by using the ratio of administered sugars. It is however not clarified whether this correction is sufficient due to possible different renal clearance of the administered sugars | Small intestine, large intestine (only if sucralose, is added) | [14-17] |
| $^{51}$Cr-EDTA (urine) | Method based on calculating the urinary excretion of orally administered test substance that reflects the non-mediated diffusion of that probe across the intestinal barrier | Not degraded by bacteria in the colon, useful marker for both the small and large intestinal permeability | Radioactivity<br>Not commonly used nowadays due to radioactivity | Influenced by renal function. Corrected in included studies: 24-h Cr-EDTA excretion = 100% of the total oral dose excreted in the urine in 24 h/creatinine | Both small and large intestine | [20-23] |
| Endotoxin level (blood), LPS (plasma) | Indirect measurement of translocation of bacterial products | High specificity | Not eligible to use among patients with inflammation in the GI tract | Unlikely to be influenced by renal function | Both small and large intestine | [18,25,26] |
| Bacterial derived DNA (16S rRNA PCR) (blood) | Direct measurement of bacterial products in blood | Optimal tool for detection and identification of bacterial isolates | Not eligible to use among patients with inflammation in the GI tract | Unlikely to be influenced by renal function | Both small and large intestine | [18,19,24] |
| Polyethylene glycols (PEG) (urine) | Method based on calculating the urinary excretion of orally administered test substance that reflects the non-mediated diffusion of that probe across the intestinal barrier. It is hypothesized that, as saccharides in sugar absorption test, molecular PEG will only cross the intestinal mucosa to the circulation in case of barrier integrity loss. Increased urinary levels of large PEGs therefore reflect an increased intestinal permeability | Biologically inert and not degraded by bacteria, thus providing information of the whole intestinal permeability | High inter- and intra-individual variations have been reported, even in healthy controls[34] | Influenced by renal function | Both small and large intestine | [28] |

AVF: Arteriovenous fistula; CAPD: Continuous ambulatory peritoneal dialysis; CKD: Chronic kidney disease; CVC: Central venous catheter; ESRD: End stage renal disease; HD: Hemodialysis; IgAN: IgA nephropathy; IgA GN: IgA glomerulonephritis; IC-GN: Immunocomplex glomerulonephritis; INS: Idiopathic nephrotic syndrome; Li: Lithium; LPS: Lipopolysaccharide; PD: Peritoneal dialysis; PEG: Polyethylene glycols; TER: Trans epithelial electrical resistance.

independent of the used method.

Figure 2 shows the results considering the mild to

**Table 2 Results considering the intestinal permeability published in the included studies**

| Ref. | Population | Study size | Marker used to assess intestinal permeability (values provided as mean ± standard deviation) | Results | Part of the intestine evaluated |
|---|---|---|---|---|---|
| Shi *et al*[18] | ESRD (both HD and non-HD) *vs* healthy controls<br>ESRD group further divided patients with bacterial DNA and without bacterial DNA in their blood samples | ESRD $n$ = 52 (HD $n$ = 22, ND $n$ = 30)<br>Controls $n$ = 10 | D-lactate (plasma)<br>Endotoxins (blood)<br>Bacterial DNA (blood) | D-lactate plasma levels higher:<br>ESRD HD *vs* controls $P$ = 0.039<br>ESRD non-HD *vs* controls $P$ = 0.044<br>HD *vs* non-HD $P$ > 0.05<br>ESRD with bacterial DNA *vs* ESRD without bacterial DNA $P$ < 0.05<br>ESRD HD with bacterial DNA *vs* ESRD non-HD with bacterial DNA $P$ > 0.05<br>Endotoxin significantly higher:<br>ESRD HD *vs* controls $P$ < 0.05<br>ESRD non-HD *vs* controls $P$ < 0.05<br>ESRD HD 0.95 ± 0.12 EU/mL<br>ESRD non-HD 0.70 ± 0.15 EU/mL<br>Controls 0.17 ± 0.10 EU/mL<br>Presence of bacterial 16S rDNA:<br>ESRD HD 6/22 patients<br>ESRD non-HD 6/30 patients<br>Controls: 0/10 patients | Large intestine<br>Mostly large intestine<br>Mostly large intestine |
| Wang *et al*[19] | ESRD patients (non-HD) *vs* healthy controls<br>ESRD group further divided patients with bacterial DNA and without bacterial DNA in their blood samples | ESRD $n$ = 30<br>Controls $n$ = 10 | D-lactate (plasma)<br>Bacterial 16s rDNA (blood) | Plasma D-lactate higher:<br>ESRD with bacterial DNA *vs* ESRD without bacterial DNA $P$ = 0.0233<br>ESRD with bacterial DNA *vs* controls $P$ =0.067<br>ESRD with bacterial DNA: 13.53 ± 1.47 μg/mL<br>ESRD without bacterial DNA: 5.71 ± 2.28 μg/mL<br>Controls: 4.82 ± 0.93 μg/mL<br>D-lactate plasma levels both ESRD groups combined: 7.274 ± 2.16 μg/mL[1]<br>ESRD: 6/30 bacterial DNA in blood<br>Controls: no bacterial DNA in blood | Large intestine |
| Bossola *et al*[24] | HD patients (AVF en CVC) *vs* healthy controls | HD $n$ = 58 (AVF $n$ = 44, CVC $n$ = 14)<br>Controls $n$ = 30 | Bacterial 16S rDNA (blood) | HD patients: 12/58 bacterial DNA in blood (= 20.7%)<br>Healthy controls: No bacterial DNA in blood<br>AVF patients 5/44 (= 15.9%)<br>CVC patients 5/14 (35.7)<br>$P$ = 0.22 | Both small and large intestine |
| McIntyre *et al*[25] | HD patients, PD patients,<br>CKD patients (stage 3-5) *vs* healthy controls | HD $n$ = 120<br>PD $n$ =25<br>CKD stage 3-5 $n$ = 90<br>Controls $n$ = 14 | Endotoxin level (blood) | Significant higher endotoxin levels in<br>HD *vs* PD $P$ < 0.008<br>Dialysis patients (HD + PD) *vs* CKD $P$ < 0.001<br>CKD *vs* controls $P$ > 0.05<br>HD patients: 0.64 EU/mL<br>PD patients: 0.56 EU/mL<br>HD + PD patients: 0.62 ± 0.37 EU/mL<br>CKD patients: 0.11 ± 0.68 EU/mL<br>Controls: Not provided | Both small and large intestine |
| Feroze *et al*[26] | HD patients, follow up for 42 mo | HD $n$ = 303 | Endotoxin level (blood) | No significant association between elevated circulating endotoxin levels and mortality<br>Mean endotoxin levels: 2.31 ± 3.10 EU/mL | Both small and large intestine |
| Zuckerman *et al*[20] | No control group<br>CAPD patients *vs* healthy controls | CAPD patients $n$ = 11 (5 with significant urine output)<br>Controls $n$ = 32 | Cr-EDTA recovery (24 h urine + dialysate) | Significant less recovery of Cr-EDTA:<br>CAPD *vs* controls $P$ < 0.0005 | Both small and large intestine |
| Szeto *et al*[27] | New PD patients *vs* IgAN patients (mild to moderate CKD) and healthy controls<br>Mean creatinine level IgAN group: 151.2 ± 116.68 μmol/L | PD $n$ = 30<br>IgAN $n$ = 10<br>Controls $n$ = 6 | LPS (plasma) | CAPD patients: Mean 0.57% (0%-1.24%)<br>Healthy controls: Mean 1.99% (0.59-3.48)<br>Significantly higher LPS levels<br>PD *vs* IgAN $P$ < 0.0001<br>PD *vs* controls $P$ < 0.0001<br>IgAN *vs* controls: Not provided<br>PD: 0.44 ± 0.18 EU/mL<br>IgAN: 0.0035 ± 0.009 EU/mL<br>Controls: 0.013 ± 0.007 EU/mL | Both small and large intestine |
| Cobden *et al*[17] | CKD patients *vs* healthy controls | CKD $n$ = 6<br>Controls $n$ = 55 | Cellobiose and mannitol recovery (urine) | No significant difference recovery cellobiose and mannitol | Small intestine |

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|---|---|---|---|---|---|
| | CKD group: Serum creatinine levels ranging from 140 to 1050 μmol/L | | | CKD *vs* controls $P > 0.05$<br>Cellobiose:<br>CKD: Recovery range 0.09%-0.44%<br>Controls: Not provided<br>Mannitol: CKD: Recovery range 12.8%-52.3%<br>Controls: Not provided | |
| Magnusson *et al*[28] | Asymptomatic uremic CKD *vs* healthy volunteers<br>Mean serum creatinine level IgAN group: 503 μmol/L, range 274-796 μmol/L | CKD $n = 9$<br>Controls $n = 6$ | PEGs (urine)<br>Computer model was used to predict the PEG recovery adjusted for eGFR | Significant lower urinary recovery of PEG's CKD *vs* controls $P < 0.05$<br>More heavy PEG's were harvest in urine CKD patients: indicating that intestinal permeability in CKD patients is more increased for larger molecules | Both small and large intestine |
| Kovacs *et al*[21] and Kovacs *et al*[23]<br>These two studies published results measured in the same patient group. Provided data by the two articles are summarized | IgAN patients (both uremic and non-uremic) *vs* healthy controls<br>Both in 1989 and after a four year follow up in 1994<br>No mean serum creatinine levels of total IgAN group provided | 1989: IgAN patients $n = 29$: (uremic $n = 24$ non-uremic $n = 5$)<br>Controls $n = 20$<br>1996: IgAN patients $n = 21$<br>No controls<br>Follow up patients further divided an analyzed in two groups; increased intestinal permeability group *vs* non-increased intestinal permeability | Cr-EDTA recovery (urine) | Significantly higher Cr-EDTA recovery in IgAN patients *vs* controls $P < 0.005$, both in 1989 and in follow up after 5 yr<br>IgAN (1989): 3.86% ± 0.29%<br>IgAN (1994): 4.57% ± 0.63%<br>Controls: 2.72% ± 0.23%<br>Only in the increased permeability group significant decrease in eGFR (Baseline eGFR 84.4 ± 6.1 mL/min *vs* 65.4 ± 8.6 mL/min after four years, $P < 0.01$) | Both small and large intestine |
| Rostoker *et al*[22] | Patients with Primary IgA glomerulonefritis and permanent proteinuria (IgA GN), INS IC-GN: Membranous + membranoproliferative) *vs* healthy controls and alcohol abusers (positive controls) | IgA GN $n = 30$<br>INS $n = 25$<br>IC-GN $n = 20$<br>Controls $n = 20$<br>Alcohol abusers $n = 5$ | Cr-EDTA recovery (urine) | Significantly higher Cr-EDTA recovery in IgA GN *vs* controls $P < 0.005$<br>INS *vs* controls $P < 0.005$<br>IC-GN *vs* controls $P < 0.005$<br>Alcohol abusers *vs* controls $P < 0.005$<br>IgA GN: Median 3.25% (0.7-17.8)<br>INS: Median 3.71% (0.82-10)<br>IC-GN: 3.40% (0.30-16)<br>Alcohol abusers: 4.9% (7-30)<br>Controls: 2% (0.4-3.9) | Both small and large intestine |
| Layward *et al*[15] | Histologically proven IgAN with proteinuria and microscopic hematuria *vs* healthy<br>No mean serum creatinine levels provided controls | IgAN patients $n = 18$<br>Controls $n = 17$ | Cellobiose/mannitol ratio (urine) | No significant difference cellobiose/mannitol ratio<br>IgA NP patients *vs* controls $P = 0.42$<br>IgA NP: 0.015 ± 0.008<br>Controls: 0.022 ± 0.015 | Small intestine |
| De Maar *et al*[14] | Renal transplant patients assessed before transplantation and in the follow up during active CMV infection and CMV negative controls | Permeability assessed before transplantation $n = 104$<br>Permeability assessed during active infection $n = 12$ (primary infections: 5, secondary infections: 7)<br>Controls (CMV-): $n = 9$ | Lactulose/mannitol ratio (urine) | L/M ratio increased during active CMV infection in 9/12 patients $P < 0.01$<br>L/M ratio active CMV infection compared to patients without CMV $P < 0.01$ | Small intestine<br>Small intestine |
| Ponda *et al*[16] | CKD stadium III patients *vs* healthy controls<br>CKD patients: mean eGFR: 51 mL/min per $1.73^2$<br>All patients and controls | CKD $n = 5$<br>Controls $n = 4$ | Endotoxin activity; expressed as fraction of the maximum response to endotoxin (plasma) | No significant difference endotoxin activity<br>CKD *vs* controls $P > 0.05$<br>CKD: 0.23 ± 0.15<br>Healthy controls: 0.20 ± 0.13 | |

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|---|---|---|
| had a vitamin D deficiency | Lactulose/mannitol ratio (urine) | L/M ratio increased with D3 therapy $P = 0.02$ (reflecting an increase in permeability)<br>L/M ratio not assessed in control group |

AVF: Arteriovenous fistula; CAPD: Continuous ambulatory peritoneal dialysis; CKD: Chronic kidney disease; CVC: Central venous catheter; ESRD: End stage renal disease; HD: Hemodialysis; IgAN: IgA nephropathy; IgA GN: IgA glomerulonephritis; IC-GN: Immunocomplex glomerulonephritis; INS: Idiopathic nephrotic syndrome; Li: Lithium; LPS: Lipopolysaccharide; PD: Peritoneal dialysis; PEG: Polyethylene glycols; TER: Trans epithelial electrical resistance. [1]Value not provided in article. Calculated as followed: sqrt((6-1)*1.47^2+(24-1)*2.28^2)/(6-1+24-1). Sp = $\sqrt{(n1 - 1) \times S1^2 + (N2 - 1) \times S2^2/(n1 - 1 + n2 - 1)}$.

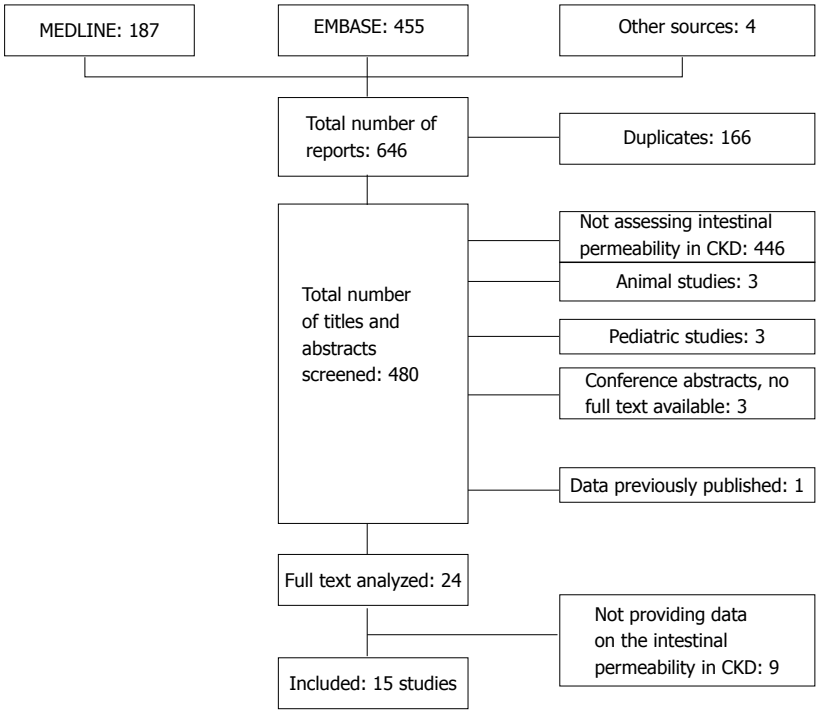


**Figure 1 Flow chart literature search.** CKD: Chronic kidney disease.

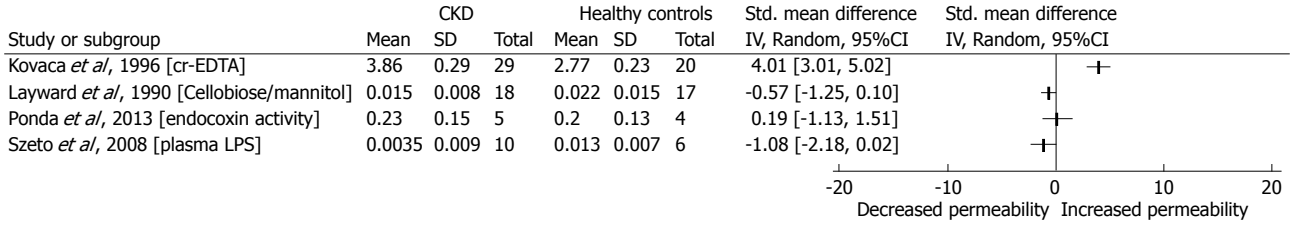


**Figure 2 Intestinal permeability mild to moderate chronic kidney disease patients (epidermal growth factor receptor 15-90) *vs* healthy controls.** CKD: Chronic kidney disease.


**Figure 3 Intestinal permeability end-stage renal disease patients (epidermal growth factor receptor < 15) *vs* healthy controls.** ESRD: End stage renal disease.

moderate CKD patients; results are less convincing, whilst some do point out a significant increased intestinal permeability other studies did not report a statistical difference.

There was one study also comparing the peritoneal dialysis (PD) and HD groups[25]. They reported increased endotoxin levels in the HD compared to the PD group ($P < 0.008$), reflecting a higher permeability in the HD

group.

## DISCUSSION

In this review we focused on the intestinal permeability, how it can be assessed and whether there is an increased intestinal permeability in CKD. The currently used methods for intestinal permeability assessment are primarily used and validated in gastroenterological research; patient with renal failure are often excluded due to the possible bias caused by the reduced eGFR. Discriminating between an altered renal clearance of the used marker and an actual increased permeability is challenging, since for most methods the influence of renal function loss has not been evaluated.

Table 1 summarizes the possible influence of renal function on each test. For most used markers and substances the influence of a decreased renal function remains unclear to a certain extent since there are no data evaluating the renal clearance of the substance.

The tests measuring bacterial products such as endotoxins and bacterial derived DNA are the least likely to be influenced by renal function as they are not actively excreted by the kidney. However these methods are not validated in renal failure. Furthermore, whether these products determined in the circulation actually represent an increased intestinal permeability is open for discussion, as the source of those bacterial products is not precisely known and could for example be the dialysate in dialysis patients. However, the hypothesis that these bacterial products in plasma are derived from the gut and are trans located into the bloodstream due to an increased intestinal permeability is supported by several findings. Shi *et al*[18] compared the endotoxin levels of plasma to the levels in the dialysate of HD patients. Endotoxin levels were markedly lower in the dialysate than in the plasma samples, suggesting another bacterial source than the dialysate. Bacterial phyla in the blood samples appeared to be similar to the samples obtained from the gut, which supports the hypothesis that these bacterial compounds are derived from the intestinal tract. Furthermore Bossola *et al*[24] reported that only five out of twelve plasma samples from HD patients contained the same bacteria as those in the dialysate, also suggestive for another source of the blood bacteria than the dialysate. They proposed the biofilm on the surface of the central venous catheter (CVC) as a possible source, as the percentages of patients with circulating bacterial DNA fragment tended to be higher in patients with CVCs (4 out of 15) than in patients with an arteriovenous fistula (AVF) (7 out of 44). This difference was however not statistically significant, which can be the result of the small number of patients. However, the species found in the patients with a CVC were *Escherichia coli* (2 patients), *Proteus mirabilis* (1 patient), *Enterococcus faecalis* (1 patient) *and Streptococcus Haemolyticus* (1 patients). These strains indicate rather an intestinal source. Interestingly and in accordance with the results published by Shi *et al*[18]: In none of the blood samples obtained from healthy controls bacterial DNA was identified.

It is likely that direct demonstration of bacterial DNA in blood through qPCR is more accurate for determining the intestinal permeability compared to endotoxin level measurement, since endotoxins are bacterial surface products while presence of bacterial DNA in blood definitively indicates bacterial presence.

Another marker that is possibly valuable in the CKD population is D-lactate. D-lactate is usually present in human blood at very low concentrations as a product of methylglyoxal metabolism, which is produced in small amounts from fat, protein and carbohydrate metabolism. However, it is also produced by bacteria in the gastrointestinal tract and absorbed in the small intestine and colon. Only 10% of D-lactate is excreted in urine[29], marking a relatively low influence of renal clearance on plasma levels. In case of bacterial overgrowth a possible increased fermentation of undigested carbohydrates to D-lactate is nevertheless an important factor that might cause bias.

Considering the sugar absorption test, various combinations of oligosaccharides (lactulose, cellobiose) and monosaccharides (mannitol, L-rhamnose) are being used. The percentage of the substance excreted in urine is defined as the urinary recovery and is often expressed as the ratio of the recovery of the administered sugars. Even though renal clearance of these sugars is assumed to be of little or no influence on the ratio since both sugars are equally affected by a reduced eGFR[17], van Nieuwenhuizen *et al*[30] observed different results in their study evaluating the influence of pre- and postabsorptive factors on the lactulose/rhamnose ratio. The urinary excretion of lactulose and rhamnose was measured in 10 healthy males after intravenous administration of different quantities of each sugars. Equal renal clearance of both sugars would assure an unchanged ratio after administering a higher dose of both sugars. The investigators found a significant ($P$ = 0.021) increase in lactulose/rhamnose ratio after administration of the high dose compared to the regular dose; a higher quantity of lactulose administration resulted in a lower recovery. These findings suggest that the process of renal clearance is different for the two sugars and thus that renal function might influence test results. Furthermore, in a study in endotoxaemic rats[31], fluid loading increased the urinary recovery of lactulose, but not of L-rhamnose. This also suggests that renal clearance of both sugars might not be equal. In conclusion, literature results on the recovery of both sugars are conflicting[30,32]. Differences in administration methods and dosage might be an explanation. The exact renal excretion of the different sugars is not clarified and thus might be affected differently when the eGFR is altered.

Furthermore, a decreased bowel motility has been reported to influence test results[33]. This test could however be valuable as a follow up method with patients being their own controls.

The studies that used $^{51}$Cr-EDTA as a marker for intestinal permeability corrected for renal function by dividing the 24 h $^{51}$Cr-EDTA excretion by the plasma creatinine level[20-23,34]. The radioactivity is nevertheless a major disadvantage that has caused this method to be considered out of date.

For the urinary recovery of different sized polyethylene glycols (PEGs), large inter- and intra-individual variations have been reported, even in healthy controls[35]. Combined with the influence of renal function on this test we consider it to be less suitable for the CKD patient population than other available methods.

Considering the results provided by the included studies, we divided the studies in categories based on the included patient population before results were compared. In our forest plots both the mild to moderate CKD patients (eGFR 15-90) and the ESRD patients (eGFR < 15) were compared to healthy controls. Seven studies have been published comparing the intestinal permeability specifically in patients with end stage renal disease, with or without dialysis, to healthy controls[18-20,24,27,36]. One of these studies included both HD and PD patients and also compared these groups.

From the studies comparing ESRD to healthy controls, three were providing sufficient data to calculate the standardized mean difference. Markers that were used in these studies were D-lactate, bacterial DNA, and endotoxins levels. Independent of the method that was used, all studies showed a significantly increased permeability in the ESRD group. These consistent results, despite the variety in the methods used, supports the hypothesis that renal failure is associated with increased intestinal permeability. The significant results published by studies measuring bacterial DNA and endotoxins are unlikely to be influenced by renal function.

The study also comparing the PD and HD groups[25] reported a significant increased permeability in the HD group compared to the PD group, $P < 0.008$. This was however the only study evaluating the difference between these two groups. All included studies including HD or PD patients reported a significant difference compared to the healthy controls. Further research is required to evaluate difference between the influence of HD *vs* PD on the intestinal permeability.

Studies assessing the intestinal permeability in mild to moderate CKD, mostly IgA nephropathy patients[15,23,27,34], yielded conflicting results. Even though some studies[21,34] reported a significantly increased permeability compared to the healthy controls, other studies could not confirm this finding[15,16,27]. Not all studies provided data on the exact renal function, but in general the eGFR was mildly decreased. This is an important difference compared to the studies assessing the intestinal permeability in end stage renal disease. Szeto *et al*[27] compared new peritoneal dialysis (PD) patients to both patients with mild to moderate CKD due to IgA nephropathy and healthy controls. Average serum creatinine levels of the IgA nephropathy group were 151.3 ± 116.2 μmol/L. He found significant higher endotoxin levels when comparing the PD patients (who suffer from a later stage of CKD) to the IgA group and the healthy controls. There was no significant difference between de IgA group and the healthy control group. This suggests that the intestinal permeability might only increase in later stages of CKD.

This systematic review outlines the lack of a gold standard to determine the intestinal permeability in the CKD patient population. Even though we aim to oppose the most reliable method, the lack of a gold standard is a limitation of this systematic review. In addition to this, unfortunately none of the included studies used more than one method to measure the intestinal permeability in CKD patients in order to be able to actually compare different methods.

In conclusion, assessing the intestinal permeability in CKD patients remains challenging as the influence of decreased renal function on the test results remains unclear. Quantitative PCR for bacterial DNA in blood and D-lactate levels in plasma seem the least likely to be influenced by a decreased eGFR. It should be noted though that also these methods have not been validated in the CKD patient population and results should still be interpret with caution[37].

However each included study measuring the intestinal permeability in patients with ESRD pointed out a significant increased permeability. Thus, it seems likely that there is a connection between renal failure and an increased intestinal permeability. How the permeability evolves in time, the possible link with (recurrent) infection(s), cardiovascular complications and prognosis of these patients has not yet been made and requires further exploration.

## COMMENTS

### Background

In the recent years numerous studies have been published evaluating the intestinal permeability in chronic kidney disease (CKD). Different methods are being used whilst the influence of a decreased renal clearance on these tests is unclear, complicating the interpretation of test results published by these studies. Her aim of this review is: (1) to determine what the best available method to measure the intestinal permeability in CKD; and (2) whether there is an increased intestinal permeability in CKD.

### Research frontiers

Noninvasive methods to measure the intestinal permeability have been used for many decades, with the first studies published in the 1950s. Only since the 90s there has however been an increasing interest in the intestinal alterations in renal failure and the possible clinical relevance of this aspect. Even though methods have been improved over the years, still none of the currently available methods has been validated in patients with renal failure. Furthermore it is still unclear whether there actually is an increased intestinal permeability in CKD and what the clinical relevance of this decreased barrier function is. Even though an increased intestinal permeability is proposed as an important prognostic factor, studies evaluating the influence of the intestinal permeability on the long-term prognosis of CKD patients have not yet been published.

### Innovations and breakthroughs

Since 2009, three studies have been published using the quantitative amount of bacterial DNA in blood as a marker for intestinal permeability in CKD patients. This method to evaluate the intestinal permeability is unlikely to be influenced by a decreased renal clearance and also points out the exact consequence

of an increased intestinal permeability; bacterial translocation into the bloodstream. This is likely to trigger an inflammatory response and could thus be an important prognostic factor for patients with renal failure.

### Applications

This review opposes the most reliable methods to determine the intestinal permeability in CKD and points out that there possibly is a link between an increased intestinal permeability and renal failure. The overview of the advantages and disadvantages of the currently available methods could help fellow researcher to determine what the most reliable method to measure the intestinal permeability in their study population. Future research is necessary specifically considering the role of the intestinal permeability as a prognostic factor in CKD. In this prospect restoration of the intestinal barrier function could also become a possible therapeutic target.

### Terminology

Even though the exact pathophysiology is not yet clarified, CKD is accompanied by a chronic inflammatory response, meaning that the immune system appears to be constantly triggered. A chronic inflammatory status is associated with many complications such as cardiovascular disease, which are in turn frequently observed in CKD.

### Peer-review

In this systematic review the authors have presented a critical analyse of the currently available methods to determine the intestinal permeability in CKD in order to guide fellow researcher in their choice of methods applicable to measure the intestinal permeability in CKD in future research projects. Furthermore the need for studies evaluating the intestinal permeability with reliable methods is emphasised, especially considering the lack of knowledge on the prognostic consequence of an increased intestinal permeability.

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