A Low Aromatic Amino-Acid Diet Improves Renal Function and Prevent Kidney Fibrosis in Mice with Chronic Kidney Disease

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Abstract

Despite decades of use of low protein diets (LPD) in the management of chronic kidney disease (CKD), their mechanisms of action are unclear. A reduced production of uremic toxins could contribute to the benefits of LPDs. Aromatic amino-acids (AA) are precursors of major uremic toxins such as p-cresyl sulfate (PCS) and indoxyl sulfate (IS). We hypothesize that a low aromatic amino acid diet (LA-AAD, namely a low intake of tyrosine, tryptophan and phenylalanine) while being normoproteic, could be as effective as a LPD, through the decreased production of uremic toxins. Kidney failure was chemically induced in mice with a diet containing 0.25% (w/w) of adenine. Mice received three different diets for six weeks: normoproteic diet (NPD: 14.7% proteins, aromatic AAs 0.019%), LPD (5% proteins, aromatic AAs 0.007%) and LA-AAD (14% proteins, aromatic AAs 0.007%). Both LPD and LA-AAD significantly reduced proteinuria, kidney fibrosis and inflammation. While LPD only slightly decreased plasma free PCS and free IS compared to NPD; free fractions of both compounds were significantly decreased by LA-AAD. These results suggest that a LA-AAD confers similar benefits of a LPD in delaying the progression of CKD through a reduction in uremic toxins production, with a lower risk of malnutrition.

Translational Statement

Uremic retention solutes (URS) such as p-cresyl sulfate and indoxyl sulfate contribute to the pathogenesis of systemic complications in patients with chronic kidney disease (CKD). Conventional dialysis provides insufficient clearance of organic solutes. Another strategy to decrease URS production could be a reduction in the dietary intake of their precursor amino acids. Here, using a mouse model of kidney failure, we show that a selective dietary reduction of aromatic amino acids (tyrosine, tryptophane and phenylalanine) in the same proportion than in a low protein diet can efficiently prevent renal fibrosis. Clinically, this work provides hypothesis generating insights for the beneficial effect of low protein diets and open the perspective of a potential benefit of the modification of “protein quality” rather than the “protein quantity” in CKD patients.

Introduction

Because nutritional status often becomes unbalanced during the course of chronic kidney disease (CKD), nutritional intervention is a mandatory component of the management of patients with CKD [1]. It has long been recognized that high protein intake has a deleterious impact on renal function in CKD patients, by increasing glomerular filtration rate, leading to progressive glomerular sclerosis [2,3]. Hence, a low protein diet (LPD) (i.e. 0.6 to 0.8 g/kg/day) is now commonly recommended for non-hemodialysis CKD patients, decreasing kidney workload and slowing renal function decline.[4] For decades, various health benefits have been attributed to protein restriction in patients with CKD, such as favorable metabolic effects, reduction of proteinuria and uremic symptoms and improvement in insulin-sensitivity [5]. One of the hypotheses put forward to explain these effects is a potential decrease in protein-derived uremic toxin concentrations, improving renal and metabolic parameters and leading to better uremia control [6]. However, if LPDs were shown to efficiently delay CKD progression, the underlying mechanisms remain poorly understood. Besides, this nutritional strategy has several drawbacks, such as a poor compliance and a potential risk of energy wasting [7].

p-Cresyl sulfate (PCS) and indoxyl sulfate (IS) are protein-bound uremic toxins produced by the gut microbiota [8]. Previous studies showed that CKD patients microbiota exhibited a loss of biodiversity and a decrease in the number of bacterial operational taxonomic units [9]. Indeed, dysregulation of intestinal microbiota, i.e. dysbiosis, could contribute to the generation of uremic toxins [10]. Indole and p-cresol the precursors of IS and PCS are produced by intestinal bacteria from the fermentation of aromatic amino acids, tryptophan and tyrosine (and also to a limited extent phenylalanine), respectively [11]. A strong association has been demonstrated between accumulation of PCS and cardiovascular damages. PCS concentrations are associated with the rise of inflammatory markers (e.g.: IL-6) and the increase in oxidative stress, through the production of reactive oxygen species.[12,13] It has also been shown that IS levels are negatively correlated with the glomerular filtration rate (GFR) and contribute to the progression of CKD.[12] Consequently, lowering uremic toxin levels in CKD patients appears to be a relevant strategy to prevent cardiovascular events and deterioration of kidney function.

Several studies clearly suggest that variations in protein quantity and/or quality induce marked changes in metabolism parameters, and substantial progress has been made in identifying potential mechanisms underlying these effects.[14] In particular, evidence suggests that enriching a diet with plant-based proteins (vs animal proteins) could reduce the risk of developing type 2 diabetes mellitus and metabolic diseases.[15] In the CKD area, recent publications suggest that plant based diet improve renal outcomes.[16,17]

The aim of the present study was to determine whether a normoproteic diet (NPD) selectively deprived in aromatic amino acids (i.e. tyrosine, tryptophan and phenylalanine), could be as beneficial as LPD in an experimental model of CKD. To test our hypothesis, we addressed two specific aims: i) To decipher whether a low aromatic amino acid diet (LA-AAD) could efficiently reduce renal fibrosis and inflammation in CKD mice ii) To assess if a tyrosine, tryptophan and phenylalanine-deficient diet is efficient to reduce IS and PCS production in CKD mice.

Results

Low aromatic amino acid diet does not alter food intake and body composition in CKD mice

In order to study in CKD mice, the specific effects of reducing dietary intake of three aromatic amino acids on uremic toxins production and renal function, we designed and constructed an amino acid-defined normal protein diet (NPD) modeled on a 14 % protein diet used as standard (AO4 diet). We also investigated two additional diets: a low protein diet (LPD) with a global decrease in amino acids content, in which 5% of calories were derived from amino acids through a uniform reduction of every amino acid in the NPD diet; and a low aromatic amino acid diet (LA-AAD) in which all aromatic amino acids were reduced by two-thirds to match the levels of the LPD, while all other amino acids were kept at the level of the NPD. All three diets were isocaloric with identical levels of dietary fat; the exact formulations of these 3 experimental diets are provided in **Table 1.**

We first ensured that LA-AAD diet had no significant impact on food intake and body composition in the control group. Energy intake was similar in all diets. Control LPD mice gained less weight than mice on NPD or LA-AAD over the course of six weeks as previously described [18] (*data not shown*). Control mice fed with NPD exhibited a higher body weight compared to CKD mice. However, no significant difference in body weight was noticed between CKD mice fed with NPD, LPD or LA-AA diet. (**Figure 1A-B**) We therefore proceeded to use control mice

with NPD as baseline for investigation of the specific contribution of reduced dietary aromatic acids in CKD mice.

Dietary intakes are summarized in **Figure 1**. Food intake (**Figure 1C**) and energy intake **(Figure 1D**) were lower in CKD-NPD mice, compared to the control group. CKD mice fed with LPD had a higher food and energy intake compared to CKD-NPD. However, there was no difference on food intake between CKD-NPD and CKD-LA-AAD. The average protein intake was 57% ($p<0.001$) and 54% ($p<0.001$) lower in CKD-LPD group, compared to CKD-NPD and CKD-LA-AAD groups, respectively (**Figure 1E**). Aromatic amino-acid intakes were significantly lower in both LPD ($p<0.0001$) and LA-AAD ($p<0.0001$) groups, compared to mice fed with NPD (**Figure 1F**). There was no statistical difference in aromatic amino-acid intake between CKD-LPD and CKD-LA-AAD groups (**Figure 1D**).

Biometric data and organ weights are presented in **Table 2**. At the end of the experimental study, the body weights of all CKD mice were lower than those of control mice. However, among CKD mice, no statistical difference in body weight was observed between the 3 specific diets. CKD-LPD had a slightly higher fat deposition that can be explained by the higher food intake but CKD-NDP and CKD-LA-AAD exhibited similar lean and fat mass. LPD and LA-AAD did not affect the nutritional status and surprisingly, CKD mice treated with LA-AAD exhibited increase albuminemia. (**Table 3**) Kidney weights were lower in CKD compared to control groups. The kidney weight of CKD-LPD mice was significantly lower than in CKD-NPD. There was no difference of kidney weight between and CKD-LA-AAD groups and CKD-NPD.

Specific reduction of dietary aromatic amino acids attenuates kidney injury in CKD mice

Eight weeks after the end of the adenine diet, circulating creatinine and urea levels in CKD-NPD were in the same range as those observed in moderate uremic patients (**Figure 2A-B**). Serum creatinine levels were significantly decreased in CKD-LPD and CKD-LA-AAD compared to CKD-NPD ($p<0.0001$), (**Figure 2A**). Blood urea nitrogen levels (**Figure 2B**) were lower in CKD-LPD as compared with that with NPD, but no significant difference was observed between CKD-NPD and CKD-LA-AAD. CKD-LA-AAD and CKD-LPD exhibited a similar reduction of urinary protein excretion reaching levels comparable to the NPD group ($p<0.05$) (**Figure 2C**). Fibrosis measured with sirius-red positive areas or through collagen 1 (COL-1) immunofluorescence staining, were significantly lower in CKD-LPD and CKD-LA-AAD groups compared to CKD-NPD group (10 ± 1 % and 13 ± 2% vs 17 ± 1%, $p<0.0001$ and $p = 0.01$ for Sirius-red and 4.3 ± 1.4 % and 5.2 ± 2.5 % vs 8.1 ± 2.9 %, $p<0.001$ and $p = 0.001$ for COL-1) (Figure 3A-C). There was no statistical difference in terms of percentage of fibrosis area between LPD and LA-AAD fed mice (Figure 3 B-C). Renal inflammation as evidenced by CD68 immunohistochemical staining is increased in CKD mice. LPD and LA-AAD almost completely abrogated the renal inflammation in CKD mice. (Figure 3D). Because adenine model mainly induces tubulointerstitial damages[19], we failed to report any difference in glomerular volume (**Figure 3E**) and no globally sclerotic glomeruli could be observed (data not shown).

We further conducted an analysis of gene expression of kidney fibrosis markers and pro-inflammatory cytokines, using real time PCR (**Figure 4**). Quantitative PCR indicated a significant reduction in the expression of renal fibrosis-related genes (*Col1a1* (collagen alpha-1 type 1), *TIMP1 (*Tissue Inhibitor of Metalloproteinase 1), *TGFb1 (*Transforming Growth Factor beta 1)) and inflammatory cytokines (*IL-6 (Interleukin 6)*, *TNFα (Tumor Necrosis Factor alpha),* and *MCP-1 (*Monocyte Chemoattractant protein 1)) in the kidneys of the CKD-LPD group compared with the CKD-NPD group. The expression of *Smad3* (Small Mothers Against Decapentaplegic 3) was not significantly different between groups (*data not shown*). LA-AAD also improved the progression of renal fibrosis

and local inflammation. In comparison with the CKD-NPD, *Col1A1* and *IL-6* expression was reduced with LA-AAD. Expressions of *TIMP1, TGFb1*, *TNFa* and *MCP-1* mRNA were not significantly different between CKD-NPD and CKD-LA-AAD but tended to be reduced. The tubular injury was evaluated by *Kim 1-* mRNA (kidney injury molecule-1) expression (a common marker of tubular injury). LA-AAD improved *Kim-1* expression compared to CKD mice fed with NPD or LPD. (**Supplementary Figure 1**)

Low aromatic amino-acid diet has no impact on metabolic parameters

Fasting glycaemia was lower in CKD mice fed with the LPD and no significant difference were observed between CKD-mice fed with the NPD and LA-AAD. (**Table 3**). In good agreement, CKD mice fed the LPD diets for six weeks again showed improved glucose tolerance, but mice fed the LA-AAD diet showed no improvement in glucose tolerance (*data not shown*).

Restriction in dietary aromatic amino acids decreases free uremic toxins concentration

To elucidate the mechanism of the renoprotective effect induced by LA-AAD and LPD, we investigated the effect of these diets on uremic toxins production. Total uremic toxin levels are presented in **Table 4** and free uremic toxin concentrations in **Figure 5**. Total PCS, total p-cresyl glucuronide (PCG), and total IS were increased in CKD mice compare to control mice but we did not observe any difference between CKD mice treated with different diets. We found no statistical differences for total indole-3-acetic-acid (IAA) concentrations between groups**.** CKD mice fed with LA-AAD diet exhibited significantly lower free PCS ($p<0.01$) and free IS ($p<0.05$) levels while LPD only modestly but significantly decreased the concentration of these uremic toxins in comparison to NPD. We failed to find any significant difference between groups for free PCG and free IAA.

Discussion

In this study, we demonstrated first that a LA-AAD exhibited a similar reno-protective effect as a LPD without the need of reducing total protein content. Indeed, the concomitant observation of kidney fibrosis, serum creatinine and urinary protein improvement provides support that reducing aromatic amino acids is efficient to metabolically mimic the action of a LPD. It is recognized from previous studies [7] that patients under a LPD are more prone to malnutrition. This experiment was not designed to explore the nutritional status in the different groups. Nevertheless, we failed to find any difference in weight in CKD groups, neither for the LPD group nor for the LA-AAD group.

In this study, we showed secondly that a specific restriction in the intake of aromatic amino acids lowered uremic toxin concentrations and mitigated inflammation that play major roles in the progression of renal damage. Surprisingly, in our CKD experimental model, LPD was able to decrease only to a limited extent the free uremic toxin concentrations, while total uremic toxin concentrations were not significantly different between each CKD diet groups. Black & al reported in a longitudinal study with 30 non-dialysis CKD patients (stage 3–4) a favorable effect of LPD to improve renal function and decrease uremic toxins concentrations after six months of nutritional intervention [6]. However, they only found a significant effect on total PCS values, but neither on IS nor on IAA serum concentration. It is very likely that lower uremic toxin concentrations could have been reached using either very low protein diet (VLPD), or aromatic amino acid corresponding concentrations. Indeed, a recent study showed that VLPD was effective to beneficially modulate gut microbiota, improving intestinal permeability and reducing serum levels of total and free IS and PCS in CKD patients [20]. We cannot exclude that in the present

study, the composition of diet by itself could have altered intestinal microbiota and uremic toxins production. For example, the total amount of starch is higher in LDP and LA-AAD than in NPD. Starch has recently been described as a prebiotic that promotes proliferation of some gut bacteria such as *Bifidobacteria* and *Lactobacilli*, increases the production of metabolites including short-chain fatty acids, which confer a number of health-promoting benefits.[21] In the present study, CKD mice exhibited a higher concentration of albuminemia without straightforward explanation. Only CKD mice fed with LA-AAD had a higher percentage of protein binding (%PB) of indoxyl sulfate *(Data not shown*). In the literature, albumin concentration (when in the normal range) did not influence the level of protein binding of uremic toxins, suggesting that other mechanism are involved such as post-translational modifications of plasma proteins (oxidation, carbamylation and glycosylation are for instance the most relevant processes)[22]. Also, we cannot eliminate that an increase of de novo albumin production which is probably less modified and binds better uremic toxins, could partially participate of the reduction of free fraction in CKD mice treated with LA-AAD. We can however not exclude that the reduction of free uremic toxins by LA-AAD could be, at least partially, a consequence of a structural changes in the proteins. Further studies are needed to explore this point.

Previous results, along with the present study, raise the issue that mechanisms, other than reduction in uremic toxin levels, could account for the positive effect of LPD. In an elegant experiment [23], Vaziri & al explored the impact of urea concentration on intestinal permeability in cultured CACO-2 cells. These results were consistent with the fact that the higher blood urea nitrogen, the higher the gut barrier was permeable, leading to endotoxemia, systemic inflammation and supposedly organ fibrosis. It is widely accepted that blood urea nitrogen is directly related to protein intake. Based on this assumption, we would have expected that the NPD but not LA-AAD group could improve renal function. However, with normoproteic intakes and blood urea nitrogen levels similar to the standard diet, the LA-AAD had comparable reno-protective effects as the LPD group, challenging this view.

Renal hemodynamics variation in response to protein feeding is a well-established process, and is nowadays being explored through the renal functional reserve concept [24]. Several studies have shown that a high protein intake was associated with a higher intra-glomerular pressure, glomerular hyper-filtration and damage to glomerular structure [2,3]. Hence, the benefit of a protein-restricted diet in patients with CKD, through this hemodynamic effect, is thought to promote preservation of kidney function. Once more, our results challenge this hypothesis, given that the LA-AAD group had a comparable protein intake to the standard diet group. Amino acids, either given through stomach tube or as an intravenous perfusion, have the same renal hemodynamic effects as an acute protein ingestion, leading to renal vasodilation and GFR rise [25–27]. Different hypotheses have been discussed regarding the physiological mechanisms that may play a role in hemodynamic variations. First, amino acids could influence renal vasodilation through metabolic substrates that influence tubular sodium reabsorption or renal oxygen consumption. Secondly, humoral mechanisms have also been discussed, based on the notion that renal hemodynamic variations occurring during post-prandial state could be directly related to the release of a humoral mediator into the systemic circulation, acting on the kidney vasodilation and glomerular filtration rate. The third hypothesis regarding amino acid impact on renal hemodynamic variations focuses on intrinsic renal mechanisms, such as tubule-glomerular feedback and tubular transport [28]. It may be possible that some specific amino acids, but not all, have significant effects on renal hemodynamics [25]. In the renal proximal tubule, amino acids are co-transported with sodium, resulting in reabsorption of both through epithelial transporters. Basolateral transporters also play an important role to regulate intracellular concentration of

different amino acids. Among others, the uniporter TAT1 (T-Type Amino Acid Transporter 1) is present in the small intestine and in renal proximal tubule epithelial cells. TAT1 acts as an aromatic amino acid efflux transporter and is important for the absorption of aromatic amino acids in the kidney and intestine [29,30]. It could therefore be interesting to measure TAT1 expression in a future study, to assess whether or not these three specific diets could have modified TAT1 expression or function.

The process by which a LA-AAD may delay renal function decline remains unclear. Tryptophan is the precursor of indoxyl sulfate, but also of serotonin and kynurenin. It has been shown that plasma tryptophan levels are decreased during CKD, proportionately to the stage of disease [31]. Accordingly, several tryptophan metabolites are also decreased in CKD patients, such as melatonin and 5-methoxytryptophan, related to a lower tryptophan hydroxylase-1 (TRP-1) expression, its main regulatory enzyme [32]. Conversely, some tryptophan metabolites are increased in CKD patients, among others IS, kynurenines, kynurenic acid and quinolonic acid [31]. Recent studies have involved tryptophan metabolites in the modulation of inflammation and fibrosis [33,34], and anti-fibrotic therapy targeting these tryptophan metabolic by-products are currently under investigation [35]. Further experimental studies are however needed to explore whether tryptophan metabolism pathways are involved in the development of kidney fibrosis.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase which participates in several key cell functions such as cellular protein synthesis and cell growth. The mTOR pathway has recently been studied in kidney pathology, as it is involved in renal hypertrophy and urinary excretion [36]. In a 5/6 nephrectomized rat model, Ohkawa & al demonstrated that LPD and the use of mTOR inhibitor (rapamycin) had similar reno-protective effects, decreasing proteinuria and improving kidney histological damage [37]. Chang & al demonstrated in a bone marrow-derived mesenchymal stem cell (BM-MSC) model that 5-methoxytryptophan (5-MTP), a recently discovered tryptophan metabolite, had the ability to inhibit pro-inflammatory mediators and protects BM-MSC against stress-induced senescence. Moreover, they showed that beneficial effects of 5-MTP was mediated through FoxO3a and mTOR pathway [38]. Because mTOR activity is regulated by aminoacid intake, it would be interesting to explore the hypothesis that qualitative modulation of aminoacid intake could influence mTOR pathway. It could be interesting, as a second step, to explore mTOR pathway and tryptophan metabolite concentrations in mice fed a LA-AAD. Likewise, we did not analyze intestinal microbiota composition of our different groups. It might be interesting to explore the abundance and diversity of the gut microbiome, according to each specific diet.

An additional issue to consider is whether such a LA-AAD could be deleterious to the body. Indeed, tryptophan metabolism has been studied for decades by psychiatrists, as it is the precursor of serotonin, the so-called “happiness neurotransmitter”. Several animal and human experiments have been carried out to test the hypothesis of tryptophan supplementation beneficial effect on depressive symptoms, with thus far ambivalent results [39]. However, the present LA-AAD was not completely deprived in tyrosine, tryptophan and phenylalanine, and intake levels were similar to the LPD. Tryptophan and phenylalanine are essential amino acids, meaning they cannot be synthesized *de novo* by the human organism, and thus they must be supplied in diet. Conversely, tyrosine is considered as a conditionally essential amino acid, which means its synthesis can be limited under certain pathological conditions. Minimal dietary amounts of each aminoacid have been well established and any attempt to define a LA-AAD in human should comply with these requirements. However, current protein intakes in Western countries do bring essential aminoacids far above the minimal requirements.

In conclusion the present study in CKD mice suggests that an experimental LA-AAD (i.e. low tyrosine, tryptophan and phenylalanine intake) has beneficial systemic effects, mimicking those of a LPD. Indeed, a LA-AAD appears efficient to delay renal function decline and prevent kidney fibrosis, without exposing to malnutrition. Significant differences between the two groups (LPD and LA-AAD) were noted such as energy intake, urea level or even inflammatory biomarkers (such as MCP-1) which could be deleterious in the long run and therefore require further studies. Future studies will be needed to determine if the specific restriction of foodstuffs containing large amounts of AAAs (e.g.: animal meats (beef, fish), eggs, lentils,…) could improve kidney function in humans.

Methods

Animals

Seventy four weeks old male C57BL/6JRj mice, were purchased from Janvier SA (Le Genest-Saint-Isle, France) and were group housed in an air-conditioned room with a controlled environment of 21 ± 0.5°C and 60–70% humidity with a 12 hours light/dark cycle. Experimental design of the study is illustrated in Fig. 6. Mice were randomly assigned in 6 groups (4–5 mice per cage). Mice were allowed a one-week period of acclimatization with free access to food and water. They were then assigned into two groups : CKD mice receiving a three weeks and a half adenine diet (0.25% w/w adenine on a A04 basis SAFE, Augy, France) to induce chronic kidney disease (CKD), and control group fed with standard diet (SAFE, Augy, France). After two week of washout, mice were divided into 6 subgroups to receive three different custom synthetic diets (SAFE, Augy, France) into each CKD and control group: LPD (5.0% proteins), a low aromatic amino-acid diet (LA-AAD) (14.0% proteins) and a normoproteic diet (NPD) (14.7% proteins) (see diet the exact composition in **Table 1**). Body weight and food intake were measured once a week, and food intake was calculated for each cage as the difference between the amount given and that removed from the cage. The food spillage, evaluated in preliminary experiments, was lower than 5% and therefore considered as negligible. All experimental procedures were performed in accordance with the guidelines laid down by the French Ministry of Agriculture (n°2013 – 118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22nd, 2010 (2010/63UE). The study protocol was approved by the local ethic committee (CETIL, Comité Ethique de l'INSA-Lyon, CNREEA n°102) on April 18th, 2018 under the reference # 11678. All experiments are conformed with the ARRIVE guidelines.

Diuresis and 24-hour proteinuria

After 6 weeks of each regimen, mice were housed for 24h into metabolic cages (Charles River laboratories), to collect 24-hour diuresis. Urine volume was determined gravimetrically, and protein concentration was measured according to the method of Bradford (Bradford reagent, Sigma Aldrich, Saint-Quentin Fallavier, France) using bovine serum albumin (BSA) as standard.

Euthanasia and necropsy

At the end of the study, mice were euthanized with sodium pentobarbital (180mg/kg intraperitoneally, Doletal). Body weight and body length were measured. Blood was drawn from cardiac puncture on heparinized syringe and centrifuged 4 minutes at 3600 x g to separate plasma. Plasma was collected, snap-frozen in liquid nitrogen and then stored at -80°C until analysis. Kidneys, liver, heart, gastrocnemius muscle and two different pads of white adipose tissue (epididymal and retroperitoneal) were dissected out. One kidney was stored for 48h in a

paraformaldehyde (PFA) 4% (w/v) solution for histology, and the second one was frozen in liquid nitrogen and kept at -80°C. All other tissues were weighted, snap-frozen in liquid nitrogen and stored at -80°C.

Biochemical analysis

Plasma creatinine and urea concentrations were measured with colorimetric assays from Cayman (Ann Arbor, USA) and Sobioda (Montbonnot, France), respectively. Plasma total cholesterol and triacylglycerol concentrations were measured with colorimetric kits Cholesterol RTU and Triglycerides PAP, respectively (Biomérieux, Marcy l'Etoile, France). Plasma albumin was assessed using BCG Albumin Assay Kit (Sigma-Aldrich, St Louis, USA). After a 5-hour fast, animals were injected i.p. with 1 g/kg D-glucose in sterile water. Blood glucose was measured prior to and 5, 10, 15, 30, 45, 60, 90, and 120 minutes after injection. Blood glucose values were determined from a drop of blood sampled from the tail using an automatic glucose monitor.

Renal histology

Kidneys were paraffin-embedded, cut and stained using hematoxylin and eosin, and Sirius red staining (Cellimaps, Dijon, France). To detect Type 1 Collagen (Col-1) and CD68, formalin-fixed sections (5µm) were deparaffinised and antigen retrieval performed by boiling sections for 20 minutes in 10 mM sodium citrate buffer (pH 6.0). Sections were then incubated with 10% normal horse serum followed by overnight incubation with primary antibodies rabbit anti-Col-1 (Abcam, Cambridge, UK: Anti-Collagen I antibody (ab254113)) and 1 hour with rat anti-mouse CD68 (Anti-CD68 antibody, ref ab125212).

Pictures of 10X of non-overlapping fields were taken with an Olympus microscope. We used Sirius red and COL-1 staining and FIJI software to quantify the area corresponding to collagen fibrils (i.e. interstitial fibrosis) for each image. We imaged the whole kidney over three entire kidney section for each mouse, with 10 to 20 non-overlapping images with a magnification of 10× were used. Control mice had mostly 18 images (therefore 6 by kidney section) while CKD mice rather had 10 images (3–4 by kidney section) because of the kidney size. Perivascular images were systematically excluded from the analysis. CD68 + cells number of each field were calculated and analyzed with ImageJ software (version 1.50, NIH).

To evaluate glomerular injury, images with a magnification of 400× were used. At least 30 PAS-stained glomerular hilar cross-sections were analyzed from each kidney sample to determine glomerular area and volume using the ImageJ software. Briefly, after identifying glomeruli with both arterioles and proximal tubule in the same cross section, the outline of the glomerular area was manually outlined for the measurement. The glomerular volume VG was calculated as $VG = (\beta/k)(A_m)^{3/2,}$ where $\beta = 1.38$ (shape coefficient for spheres), $k = 1.1$ (size distribution coefficient), and A_m is the surface area of the glomerulus.

Measurement of uremic toxins

Free and total concentrations of uremic toxins were quantified by ultra-high performance liquid chromatography with ultraviolet and fluorescence detection (UPLC-UV/FLD) as previously described[40]. In statistical analysis, values comprised between LOD and LOQ were replaced by (LOD + LOQ)/2.

Analysis of gene expression

Kidney tissue was crushed into liquid nitrogen, and total RNA was extracted using TRIzol Reagent, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Purity and concentration of RNA were

determined using NanodropOne (Ozyme) and quality checked using Bioanalyser (Agilent). First-strand cDNAs were synthesized from 1 µg of total RNAs using PrimeScript RT kit (Ozyme). Real-time PCR assays were performed with Rotor-Gene 6000 (Qiagen) using SYBR qPCR Premix Ex Taq (Ozyme). TATA-box binding protein (TBP) was used as reference gene to normalize the results. Primers sequences are listed in **Supplementary Table 1**.

Statistical analysis

Data are expressed as mean ± SEM. All statistical analyses were performed using R version 3.5.1 and GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). D'Agostino & Pearson test was used to assess normality. Student t test or Mann-Whitney U test were used for simple comparison, whereas ANOVA or Kruskall-Wallis analyses were performed for multiple comparisons. Welch's correction was applied in case of variance inhomogeneity. Multiple post-hoc comparisons using Tukey test and Dunnett correction were performed to identify significant differences between groups. The different groups were compared to the CKD mice with NPD. A p value < 0.05 was considered as statistically significant.

Declarations

DISCLOSURES

None

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AUTHOR CONTRIBUTIONS

CB, BB and EB carried out the experiment. CB contributed to the interpretation of the results, wrote the manuscript with support. CS and LK conceived the original idea and were in charge of overall direction and planning. GG provided the uremic toxin quantifications. SC and A V-M performed histological staining. SP performed PCR extraction. CP aided in interpreting histological results. Cl. P helped in animal sacrifice. LK, CS and DF supervised the findings of this work. CB, CS and LK wrote the article. All authors reviewed and approved the final version. LK is the guarantor of this work and accept full responsibility for the conduct of the study.

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Tables

Table 1. Food composition and energy value of the different diets

	NPD	LPD	LA-AAD
Starch (g/kg)	394.4	520.5	407.8
Maltodextrin (g/kg)	142.0	142.0	142.0
Sucrose (g/kg)	110.0	110.0	110.0
Soya oil (g/kg)	70.0	70.0	70.0
Cellulose (g/kg)	50.0	50.0	50.0
Choline bitartrate (g/kg)	2.5	2.5	2.5
Aminoacid composition g/kg			
Alanine (Ala)	4.93	1.62	4.93
Arginine (Arg)	6.08	1.98	6.08
Aspartic Acid (Asp)	11.56	3.78	11.56
Cysteine (Cys)	3.69	1.23	3.69
Glutamic Acid (Glu)	36.63	12.09	36.63
Glycine (Gly)	3.05	1.00	3.05
Histidine (His)	5.00	1.63	5.00
Isoleucine (Ile)	9.02	2.95	9.02
Leucine (Leu)	15.29	4.99	15.29
Lysine (Lys)	13.41	3.68	13.41
Methionine (Met)	4.98	1.65	4.98
Phenylalanine (Phe)*	**8.43**	**2.75**	**2.74**
Proline (Pro)	18.02	5.95	18.02
Serine (Ser)	10.15	3.33	10.15
Threonine (Thr)	7.09	2.33	7.09
Tryptophan (Trp)*	1.88	0.62	0.59
Tyrosine (Tyr)*	9.31	3.07	3.07
Valine (Val)	11.56	3.78	11.56
Protein (%)	**14.7**	**5.0**	**14.0**
Carbohydrates (%)	13.8	13.8	13.8
Fat (%)	7.1	7.2	7.1
Fibers (%)	3.6	3.6	3.6
Starch (%)	47.6	59.0	48.8

Minerals (%)	2.3	2.3	2.3
Energy density (kcal/g)	3.89	3.84	3.89
Aromatic amino acids (mg/g)	**19.62**	**6.44**	**6.40**

Data are expressed as percentage for food composition and kcal/g for energy value. *Aromatic amino acids including tyrosine, tryptophan and phenylalanine are displayed in bold. Note that the low aromatic AA diet concentration of aromatic amino acids was similar to that low protein diet.

Abbreviations: NPD: normoproteic diet, LPD: low protein diet; LA-AAD: low aromatic amino-acid diet

Table 2. Biometric data

Variable	**Control mice**			**CKD mice**								
Diet	**NPD**			**NPD**			**LPD**			**LA-AAD**		
N	9			11			8			13		
Biometric data												
BW (g)	29	±	1***	22	±	0.1	20	±	1	21	±	0.1
BL (cm)	9.5	±	0.1***	8.7	±	0.1	8.6	±	0.1	8.6	±	0.1
White adipose tissue weights, mg/10g BW												
Total WAT	297	±	24***	208	±	14	333	±	14*	228	±	9
rWAT	55	±	6***	33	±	2	67	±	3*	35	±	2
eWAT	242	±	18***	175	±	12	266	±	12*	194	±	7
Organ weights, mg/10g BW												
Heart	45	±	1***	55	±	3	50	±	1	51	±	1
Gastrocnemius	48	±	2*	57	±	2	50	±	2	54	±	1
Liver	413	±	8	395	±	14	347	±	8	374	±	11
Kidneys	119	±	2***	86	±	3	54	±	2*	76	±	1

Data are expressed as mean ± SEM. Abbreviations: BL: body length, BW, body weight, NPD, normoproteic diet, LPD, low protein diet, LA-AAD, low aromatic amino-acids, WAT, white adipose tissue. *$p < 0.05$, ***$p < 0.001$ vs CKD-NPD (ANOVA and Dunnett post hoc test).

Table 3. Biochemical data

Variable	Control mice			CKD mice								
	NPD			NPD			LPD			LA-AAD		
Fasting glucose (mmol/L)	4.1	±	0.2	4.3	±	0.2	3.2	±	0.1*	4.5	±	0.1
Fed glucose (mmol/L)	8.2	±	0.4	8.8	±	0.5	6.8	±	0.5	8.1	±	0.5
Total cholesterol (mmol/L)	4.0	±	0.3	3.3	±	0.4	3.6	±	0.1	3.9	±	0.2
Triglycerides (mmol/L)	2.3	±	0.2	2.7	±	0.2	1.5	±	0.2*	3.0	±	0.2
Plasma proteins (g/L)	46.7	±	1.9	45.7	±	1.5	47.8	±	4.4	46.9	±	1.9
Serum albumin (g/L)	15.8	±	0.8	18.9	±	0.5	18.1	±	0.5	21.1	±	0.8
Diuresis (mL/24h)	1.2	±	1.0***	3.2	±	1.6	2.0	±	0.9*	1.9	±	0.9*

Data are expressed as mean ± SEM for n = 5-12 animals in each group. Abbreviations: NPD, normoproteic diet, LPD, low protein diet, LA-AAD, low aromatic amino-acids, CKD, chronic kidney disease. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD (ANOVA and Dunnett post hoc test).

Table 4. Serum concentration of total uremic toxins (µmol/L)

	Control mice			CKD mice								
Variable	NPD			NPD			LPD			LA-AAD		
PCS	0.6	±	0.2**	4.5	±	0.9	3.6	±	1.1	2.5	±	0.7
PCG	0.1	±	0.1 *	0.6	±	0.3	0.6	±	0.3	0.2	±	0.1
IS	8.2	±	1.2 ***	37.5	±	3.1	28.6	±	2.3	42.6	±	4.7
IAA	0.2	±	0.1	0.2	±	0.1	0.3	±	0.1	0.3	±	0.1
HA	290	±	52	284	±	48	298	±	38	276	±	14
Uric acid	64.5	±	9.8	87	±	19	78	±	25	64	±	13

CKD: chronic kidney disease. PCS: P-cresyl sulfate. PCG: p-cresyl glucuronide. IS: indoxyl sulfate. IAA: indole-3-acetic-acid. HA: hippuric acid. Data are expressed as mean ± SEM. NPD: normoproteic diet. LPD: low protein diet. LA-AAD: low aromatic amino-acids diet. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD (ANOVA and Dunnett post hoc test).

Figures

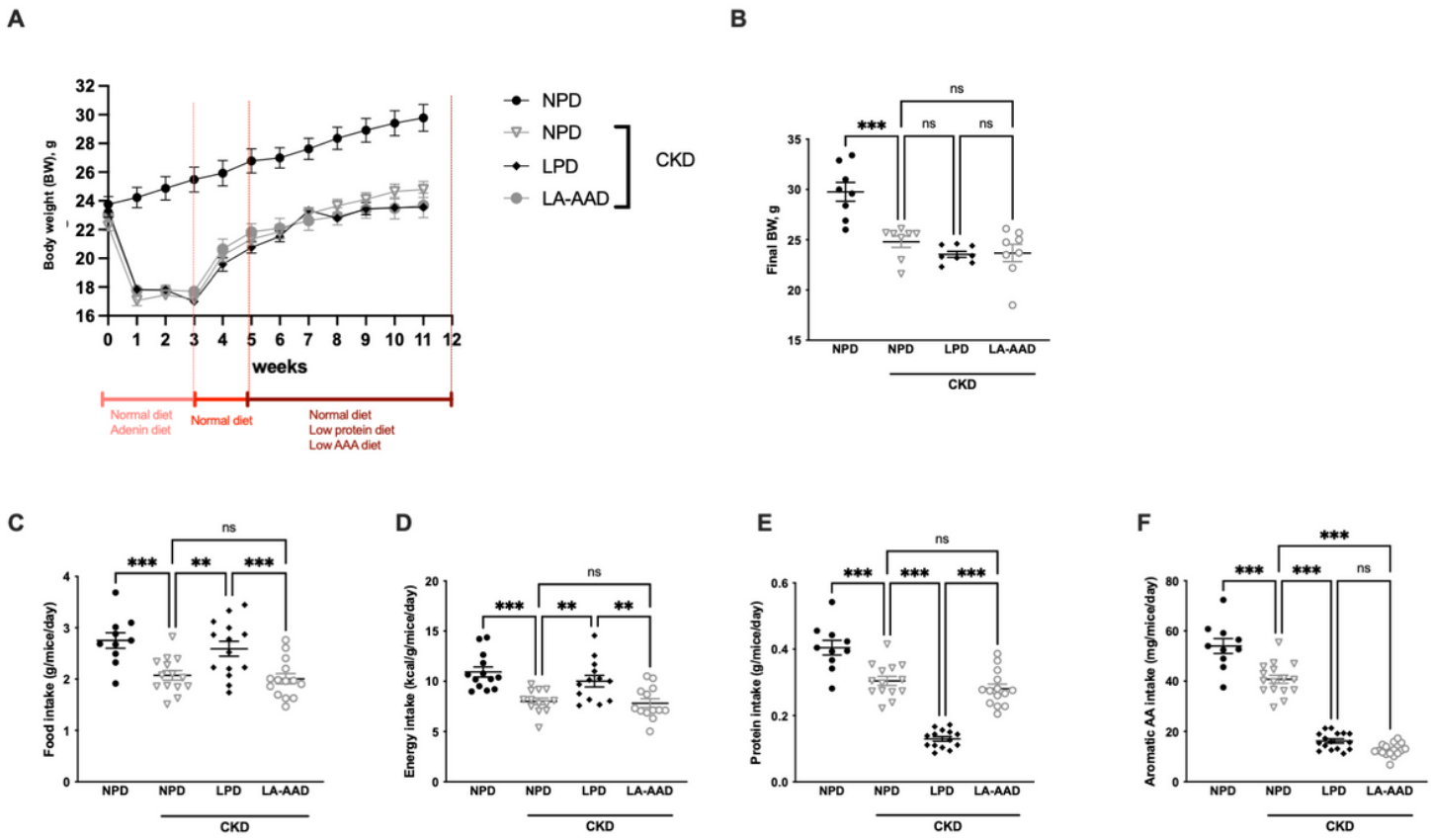

Figure 1

Food, energy, protein and aromatic amino acids intakes according to each specific diet and renal condition Body weight evolution (A), final body weight (B), food intake (C), energy intake (D), protein intake (E) and aromatic amino-acid intake (F) in control mice fed with normoproteic diet (NPD) and chronic kidney disease (CKD) mice fed with NPD, low protein diet (LPD) or low aromatic amino-acid diet (LA-AAD). Data are presented as mean ± SEM for n = 10-13 animals in each group. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD; (ANOVA and Dunnett post hoc test).

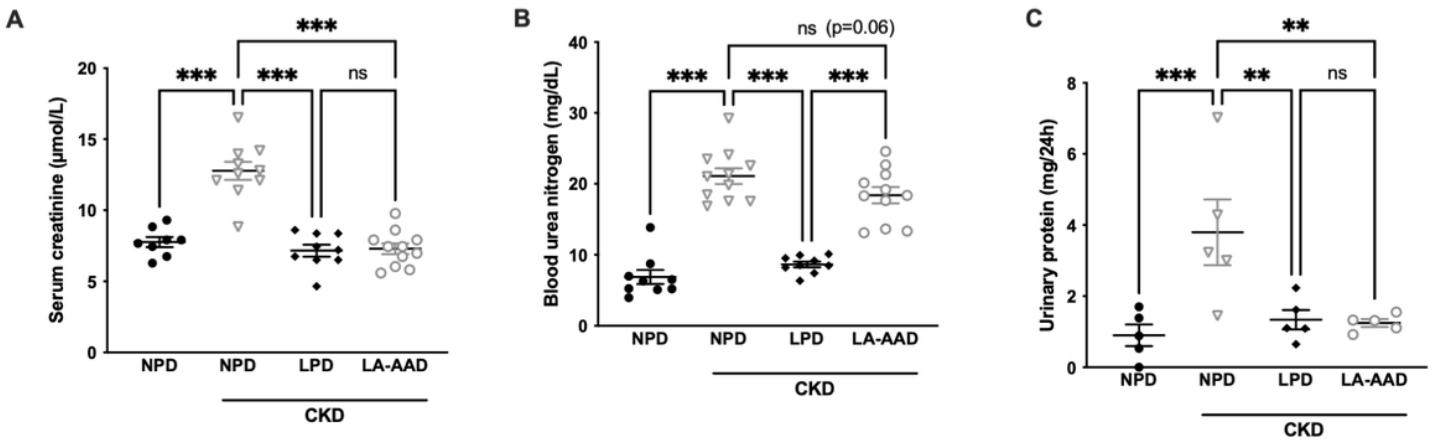

Figure 2

Kidney function and urinary protein excretion in control and CKD mice Serum creatinine (A), blood urea nitrogen (B) and urinary proteins (C) in control and CKD mice fed with normoproteic diet (NPD), low protein diet (LPD) or low aromatic amino-acid diet (LA-AAD). Data are expressed as mean ± SEM for n = 9-11 animals in each group. Proteinuria was only measured on 5 animals per groups. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD; (ANOVA and Dunnett post hoc test).

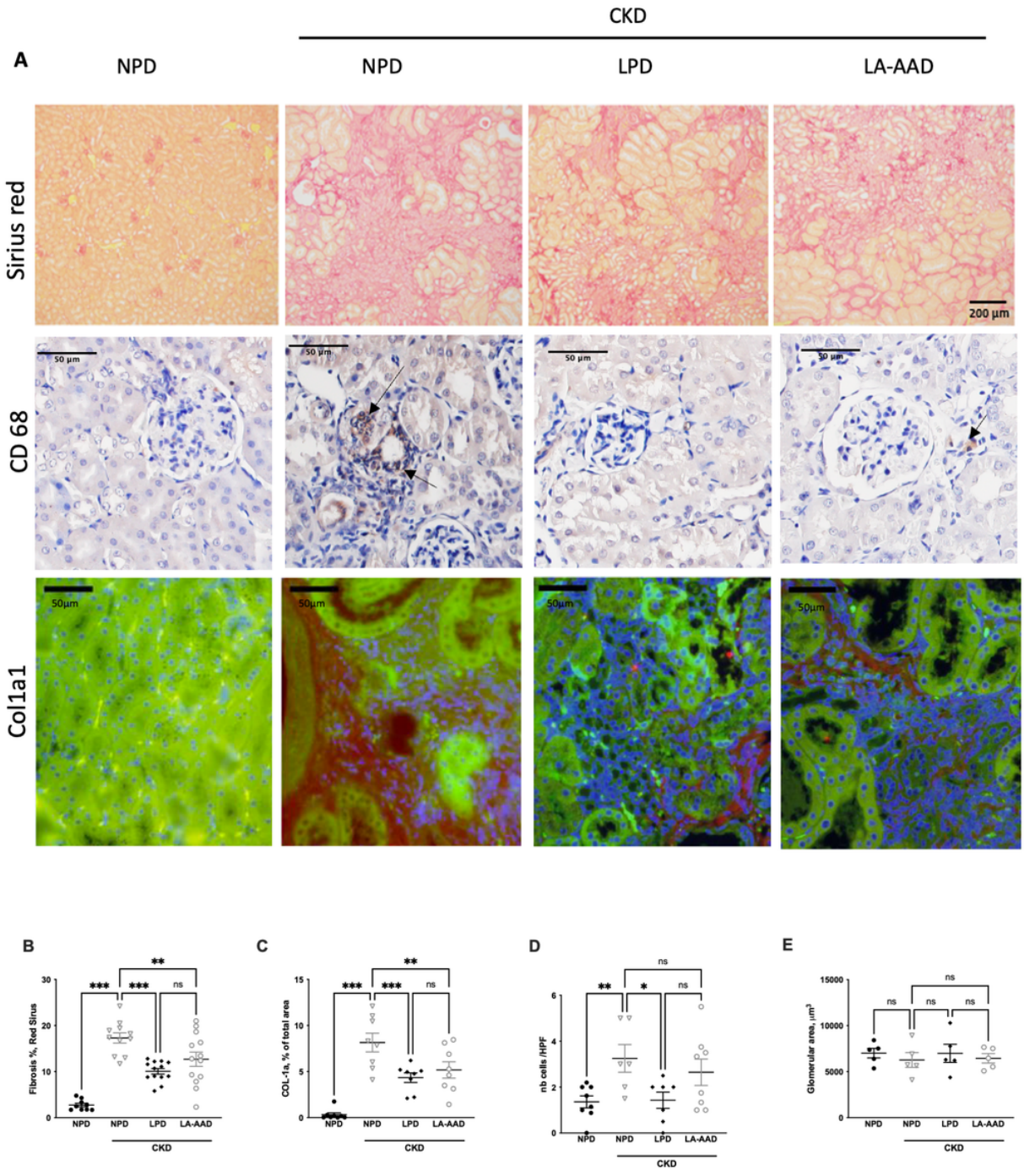

Figure 3

Evaluation of kidney fibrosis, glomerulosclerosis and kidney inflammation in control and CKD mice (A) Representative images of kidney sections stained with Sirius Red viewed under bright field x10. Scale bar represents 200 µm. Immunohistochemistry staining for CD68 and immunofluorescence staining for Col1a1. Scale bar represents 50 µm. (B) Sirius red morphometric and (C) Col1a1 evaluation in control and CKD mice treated with normoproteic diet (NPD), low protein diet (LPD) and low aromatic amino-acid diet (LA-AAD). (D) Tubular cell damage scores obtained by CD68 quantification (E) Glomerular area was quantified using ImageJ software and expressed as glomerular volume. CKD: chronic kidney disease. Data are expressed as mean ± SEM for n = 9-13 animals in each group. **p < 0.01, ***p < 0.001 vs CKD-NPD; (ANOVA and Dunnett post hoc test)..

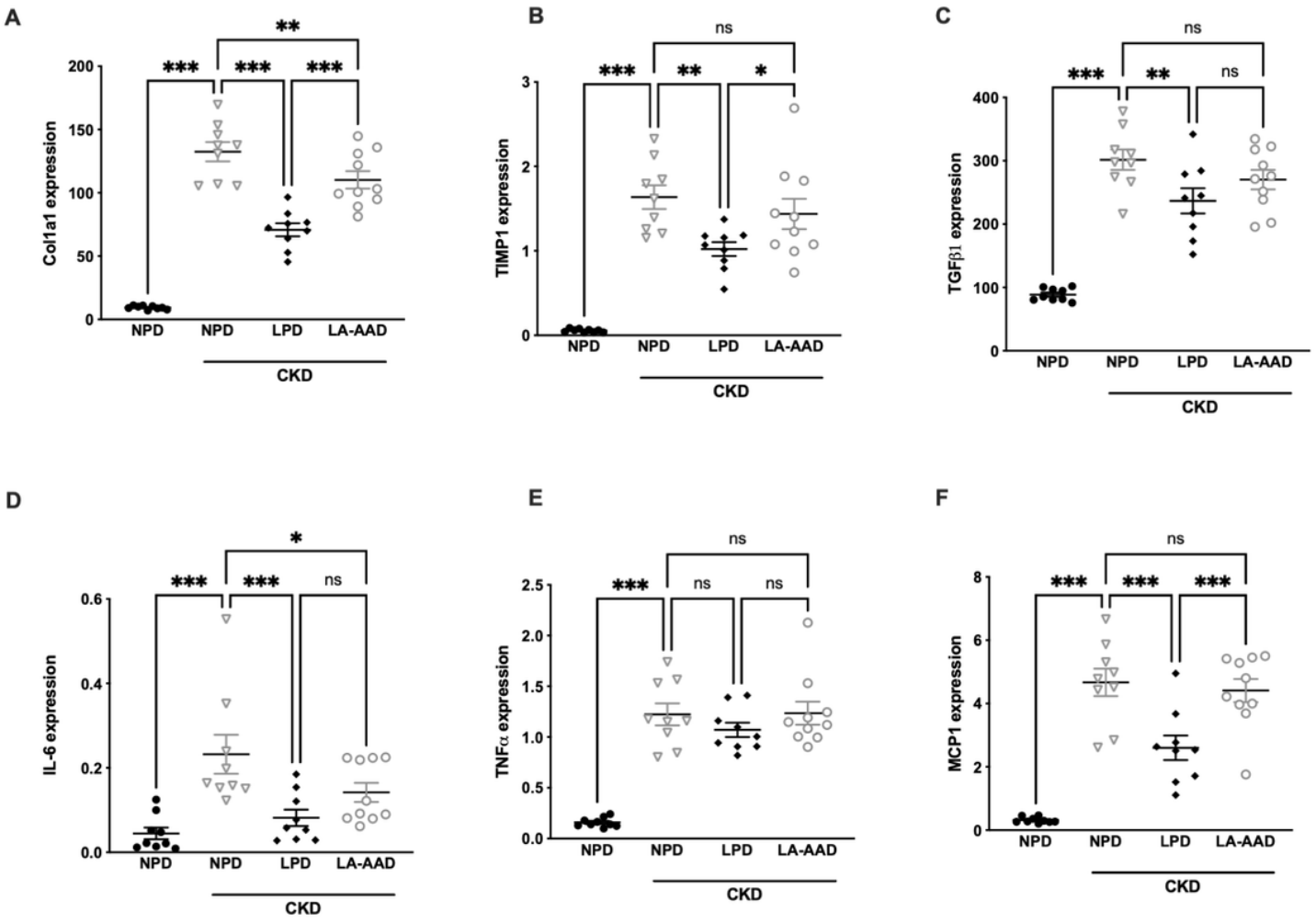

Figure 4

Gene expression of fibrosis and pro-inflammatory markers in kidney Effects of normoproteic diet (NPD), low protein diet (LPD) and low aromatic amino-acid diet (LA-AAD) on relative mRNA expression of (A) Col1a1 (collagen alpha-1 type 1), (B) TIMP1 (Tissue Inhibitor of Metalloproteinase 1), (C) TGF1 (Transforming Growth Factor beta 1), (D) IL-6 (Interleukin 6), (E) TNFα (Tumor Necrosis Factor alpha) and (F) MCP-1 (Monocyte Chemoattractant protein 1) in control and CKD mice . TBP (TATA-Box Binding Protein) was used as reference gene to normalize the results. Data are expressed as mean ± SEM for n = 9-10 animals in each group. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD; (ANOVA and Dunnett post hoc test).

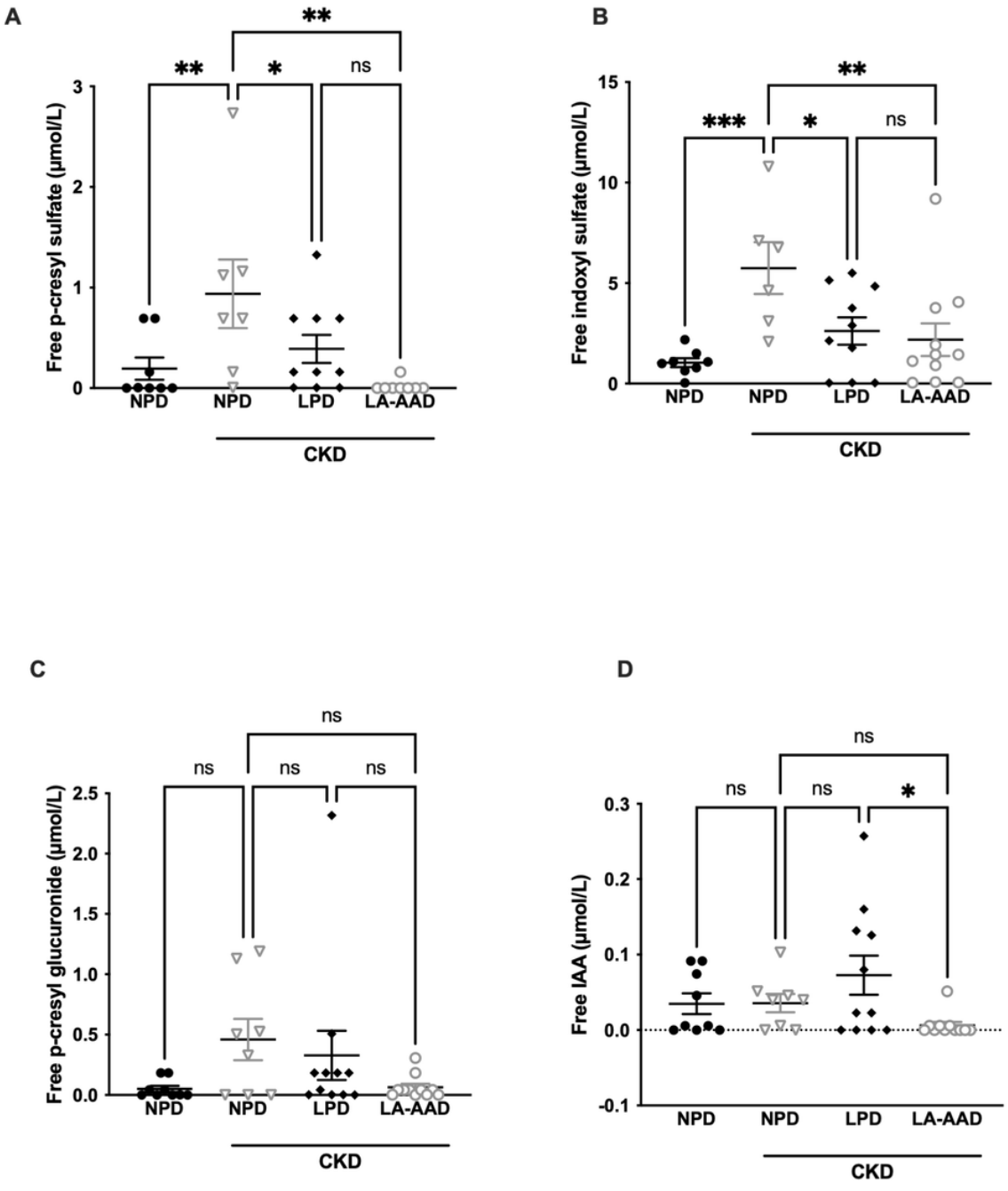

Figure 5

Free uremic toxin concentrations in control and CKD mice Effects of normoproteic diet (NPD), low protein diet (LPD) and low aromatic amino-acid diet (LA-AAD) on free (A) p-cresyl sulfate, (B) indoxyl sulfate, (C) p-cresyl glucuronide and (D) indole 3-acetic-acid (IAA) concentrations in control and CKD mice. Data are expressed as mean ± SEM for N= 8-11 animals in each group. *p < 0.05, **p < 0.01, ***p < 0.001 vs CKD-NPD; (ANOVA and Dunnett post hoc test). CKD: chronic kidney disease.

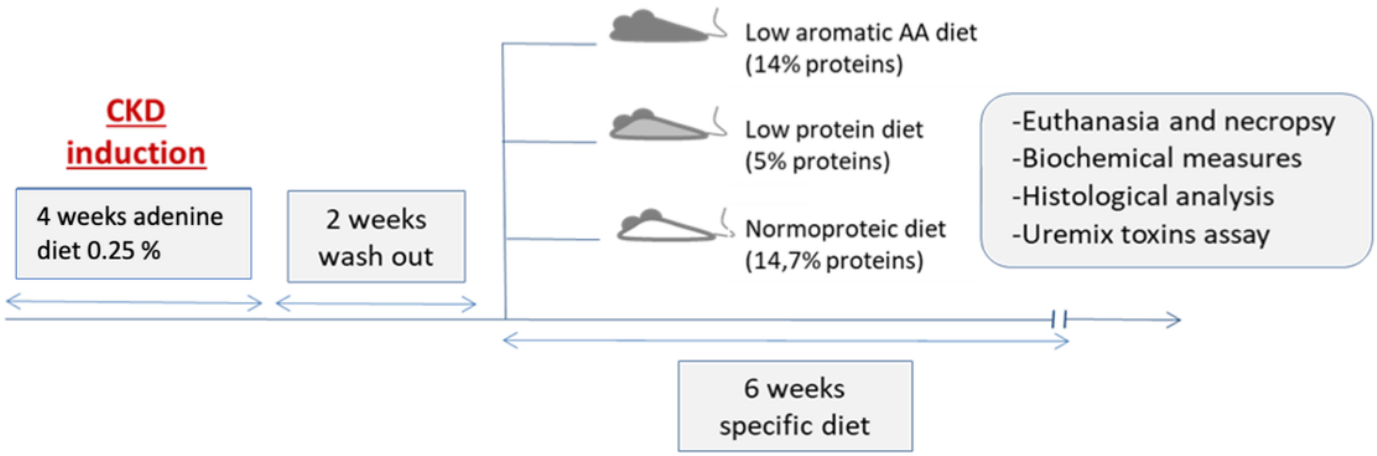

Figure 6

Experimental design of the study Schematic representation of the experimental study. Half of the C57BL/6J mice were fed with an adenine diet 0.25% for 4 weeks to induce chronic kidney disease (CKD). After 2 weeks of washout, mice were divided into 6 subgroups to receive either a low protein diet, a low aromatic amino-acid diet or a normoproteic diet until terminal sacrifice after 6 weeks of specific diet.

Supplementary Files

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- BarbaalsupplementaryR1.docx
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