Tissue-specific roles of the pattern recognition receptors NLRP3, NLRX1 and TLR9 in sterile inflammatory kidney disease

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Chapter 2

Nlrp3 is a Key Modulator of Diet-Induced Nephropathy and Renal Cholesterol Accumulation

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CHAPTER 2

Abstract

Metabolic Syndrome (MetSyn) is a major health concern nowadays and associates with the development of kidney disease. The mechanisms linking MetSyn to renal disease have not yet been fully elucidated but are known to involve hyperuricemia, inflammation and fibrosis. As the innate immune receptor Nl rp3 is an important mediator of obesity and inflammation, we investigated the role of Nl rp3 on the development of MetSyn-associated nephropathy by administering wild-type or Nl rp3-deficient mice a Western-style diet or fructose water. Here, we show that Nl rp3 is crucial for the development of Western-style diet-induced renal pathology as reflected by the prevention of renal inflammation, fibrosis, steatosis, micro-albuminuria and hyperuricemia in the absence of Nl rp3. A plausible driver of pathology, the Nl rp3-dependent cytokine IL-1β was not increased in the kidney. Interestingly, we found enhanced renal cholesterol accumulation, a well-known driver of renal pathology, during MetSyn which was Nl rp3-dependent. Furthermore, we investigated the role of Nl rp3 and fructose water on the development of MetSyn and kidney function since fructose is an important driver of obesity and kidney disease. Surprisingly, we observed that fructose did not induce MetSyn but irrespective of this, did induce renal inflammation which was Nl rp3-dependent. In conclusion, we show a crucial role for Nl rp3 in mediating renal pathology in the context of diet-induced MetSyn.
Introduction

Metabolic Syndrome (MetSyn) is defined as the presence of any three of the following five traits: abdominal obesity, hypertriglyceridemia, low HDL cholesterol, insulin resistance and increased blood pressure. MetSyn is a major risk factor for the development of cardiovascular disease (1), type 2 diabetes (2) and renal disease (3–5). In addition, a Western diet, rich in cholesterol and fatty acids, and fructose consumption closely correlates with the epidemic rise in obesity and MetSyn (6,7) and both are able to induce renal disease albeit via different mechanisms (8–11). Recently, a meta-analysis confirmed that MetSyn imposes an increased relative risk on the development of chronic kidney disease (CKD) (12). CKD is a largely irreversible disease characterized by tubulo-interstitial inflammation, fibrosis and glomerulosclerosis and associates with hyperuricemia. It is important to delay or halt these characteristics at an early stage to prevent the development of CKD. In this light it is important to elucidate the molecular mechanisms linking MetSyn and renal disease.

MetSyn is characterized by a state of systemic low grade chronic inflammation (13). The innate immune receptor Nod-like receptor protein 3 (Nlrp3) is a cytosolic protein important in inducing inflammation (14–16) through activation of Caspase-1 and maturation of the pro-inflammatory cytokine interleukin-1β (IL-1β). Nlrp3 deficiency protects against the development of obesity, insulin resistance and associated inflammation after high-fat diet feeding (17–20). We and others have found that Nlrp3 deficiency is able to prevent renal inflammation (15) or fibrosis in a model for respectively ischemia reperfusion injury and unilateral urethral obstruction. Furthermore, circumstantial evidence links the Nlrp3-inflammasome upregulation to MetSyn-associated nephropathy as well (21,22). The role of Nlrp3 on the development of MetSyn-associated renal pathology remains however unclear.

Therefore, the aim of our study was to investigate the potential of fructose versus a Western diet to induce MetSyn-associated nephropathy and to elucidate the role of Nlrp3 during development of MetSyn-associated nephropathy. For this purpose, we exposed wild-type and Nlrp3-deficient (Nlrp3KO) mice to a Western-style diet with control water (Western diet) or a control diet with fructose water (Fructose water). Next, we analyzed metabolic parameters and focused on renal pathology and underlying mechanisms.
CHAPTER 2

Material & Methods

Mice
The generation of Nlpr3-deficient mice was described previously (57). Nlpr3-deficient mice were backcrossed onto the C57BL/6 genetic background for nine generations. Male Nlpr3KO and wild-type C57BL/6 (Charles River) were subjected ad libitum to either a control diet (#4021.84, AB Diets, providing 11 energy-% in the form of fat), a western diet (#4021.84, AB Diets; a 43 energy-% high fat diet containing 0,15% cholesterol) (see Supplementary Table 1) or to plain water or water enriched with 15% fructose (w/vol) (Sigma Aldrich) (58,59). Dietary intervention continued for 16 weeks after which mice were anaesthetized and sacrificed by cardiac puncture. Prior to sacrifice, mice were starved for 4 hours to obtain fasting blood glucose levels (Bayer Contour glucose meter). Blood was collected by heart puncture. Organs were harvested, weighed, dissected and both formalin-fixed for 24h or snap frozen using liquid nitrogen. All procedures were approved by the Institutional Animal Welfare Committee of the Academical Medical Center Amsterdam.

Organ Homogenates
Organ homogenates were prepared by dissolving organs in Greenberger Lysis buffer (75 mM NaCl, 7.5 mM Tris, 0.5 mM MgCl₂.H₂O, 0.5 mM CaCl₂, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Sigma Aldrich) and mechanically disrupted using a tissue homogenizer (Polytron PT1200E).

ELISA
Cytokines were measured using an enzyme-linked immunosorbent assay for MCP-1, KC, IL-1β, IL-6 and TNF-a (R&D Systems) according to the manufacturers’ instructions. Samples were normalized for the amount of protein using a Bradford assay (Bio-Rad) with bovine serum albumine as a reference. Plasma insulin was determined using the ultra-sensitive mouse insulin kit (Crystal Chem Inc) following manufacturers’ instructions.

Immunohistochemistry
As described previously, formalin-fixed paraffin-embedded kidney sections were stained for macrophages and granulocytes (60) or T cells (61). ICAM-1 and collagen type 1 expression was visualized using the following antibodies, respectively goat α-mouse ICAM-1 (R&D Systems) and rabbit α-mouse collagen
type 1 (GeneTex Inc). Briefly, sections were boiled in 0.01 M citrate buffer (pH 6) for collagen type 1 or in 10 mM Tris, 1 mM EDTA pH 9 for ICAM-1. Secondary antibodies used for collagen type 1 were power rabbit poly-HRP (Dako) or swine α-goat HRP (Dako) for ICAM-1. Staining was visualized using DAB (Sigma Aldrich) and counterstained using Methyl Green (Sigma Aldrich).

**Quantification of immunohistochemistry**

Vacuolated tubuli were quantified in 10 randomly chosen medium power fields (MPF) (200x) of the cortex. Tubuli which contained >75% vacuoles and were fully visible in the MPF were counted. Peri-glomerular collagen type 1 accumulation was quantified in 10 randomly chosen MPFs (200x) in the renal cortex. Glomeruli which peri-glomerular border was >50% collagen type 1-positive were counted. Quantification of F4/80 immunohistochemistry was performed by scanning whole sections using the Dotpro Slidescanner and subsequently quantifying pixels that had spectra specific for positive pixels using the processing software ImageJ version 1.43u.

**Biochemical analysis**

Plasma urea, creatinine, uric acid, sodium and chloride and urinary creatinine were measured using standardized clinical diagnostic protocols of the Academical Medical Center Amsterdam. Albumin (Bethyl Laboratories) was measured following manufacturers’ instructions in urine. Non-esterified fatty acids (DiaSys Diagnostics Systems), triglycerids (Biolabo Reagents), phospholipids (Biolabo Reagents) and cholesterol (Biolabo Reagents) were measured following manufacturers’ instructions in plasma and kidney homogenates.

**Histochemistry**

For visualization of phospholipids, frozen sections of 8 µm were cut and fixed with 1% glutaraldehyde and stained with Nile Red (1 µg/ml) (Sigma Aldrich). Fluorescence for Nile Red was observed with a K3 filter (Leica DM500B). Spectral unmixing was achieved using Nuance software. Presence of cholesterol crystals was investigated using electron microscopy (EM) and polarizing light microscopy. For EM, frozen kidney material was fixed in Karnovski and post-fixed with 1% osmiumtetroxide. The tissue samples were block-stained with 1% uranyl acetate, dehydrated in dimethoxypropane and embedded in epoxyresin LX-112. Sections were stained with tannic acid, uranyl acetate and lead citrate
and examined using a Philips CM10 transmission electron microscope. Frozen kidney sections of 8µm were used for polarizing light microscopy.

**Quantitative real time-PCR**

Total RNA was isolated from frozen sections using Trizol (Life Technologies) following the recommended manufacturers' protocol. Complementary DNA (cDNA) was made by ligation of oligo-dT primers and subsequent polymerization using Taq DNA polymerase (Invitrogen). Finally, cDNA was quantified in real-time on a Roche LightCycler 480 (Roche Diagnostics) using LightCycler 480 DNA SYBR Green (Roche Diagnostics). To quantify gene expression, TATA-binding protein expression (TBP) was used as an endogenous reference.

**Immunoprecipitation of IL-1β**

To detect IL-1β, we immuno-precipitated 1 mg of protein derived from snap-frozen kidneys by lysis of renal tissue with isolation buffer (20 mM Tris-Cl pH 7.4, 135 mM NaCl, 1.5 mM MgCl2, 10% Glycerol, 1% Triton X100). Subsequently, all IL-1β was isolated using an anti-IL-1β rat IgG1 monoclonal antibody (MAB401, R&D Systems) followed by precipitation using Protein G-Sepharose beads (Biovision). Subsequently, all precipitated IL-1β was visualized with Western blot using pre-cast Tris-HEPES-SDS 4-20% gradient gels (Thermo Scientific) followed by anti-IL-1β polyclonal goat IgG (BAF401, R&D Systems) and streptavidin-poly HRP (Sanquin). As a positive control for immuno-precipitation or Western blot, we added 100 pg of recombinant IL-1β (R&D Systems) to 1 mg of protein or 50 pg of recombinant IL-1β (R&D Systems) respectively. A band at 18 kDa was interpreted as active IL-1β.

**Statistics**

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the non-parametric Kruskal-Wallis test with Dunn’s correction to test for multiple group comparison. The non-parametric Mann Whitney test was used for two group comparisons. Correlation between variables was determined using Spearman. For all analyses, p<0.05 was considered significant.
Results

Metabolic Syndrome traits in Wild type and Nlrp3KO mice

We found that Nlrp3KO mice given Fructose water or a Western diet showed increased caloric intake versus corresponding wild-type mice (Fig 1A) due to altered food and water intake (Supplemental Figure 1A,B). Accordingly, weight gain was significantly increased in these mice (Fig 1B). Weight gain relative to a control diet was calculated to correct for differential weight gain in mice fed a control diet. Nlrp3KO mice fed Fructose water and not Western diet-fed Nlrp3KO mice showed increased relative weight gain compared to corresponding wild-type mice (Fig 1C). Examination of several other organ and tissue weights (Supplemental Figure 1 C-F) revealed that a Western diet induces adiposity in both strains whereas only Nlrp3KO mice fed Fructose water show increased epididymal white adipose tissue (WAT) (Fig 1D). Plasma triglycerides were reduced in all three dietary groups of Nlrp3KO versus wild-type mice (Fig 1E). Finally, insulin resistance was increased in both strains after feeding a Western diet (Fig 1F). In summary, a Western diet induces MetSyn in both strains whereas Fructose water induces it only in Nlrp3KO mice.

Nlrp3 deficiency reduces MetSyn-associated nephropathy

We next investigated the effect of the different diets on Nlrp3 and Caspase-1 gene expression in kidney. Renal Nlrp3 gene expression was profoundly induced in wild-type mice by a Western diet but not Fructose water versus a control diet (Fig 2A). Caspase-1 renal gene expression was similar in all groups (Fig 2B). Although Fructose water and a Western diet did not induce elevated urea or creatinine levels in both mouse strains (Fig 2C,D), hyperuricemia was strongly induced in wild-type mice after Fructose water and a Western diet (Fig 2D). Nlrp3 deficiency profoundly prevented the development of hyperuricemia (Fig 2D) and in addition led to lower urea levels in all dietary groups (Fig 2C). Micro-albuminuria predisposes to CKD (23). We observed that Nlrp3 deficiency prevented micro-albuminuria development in Western diet-fed mice compared to control diet-fed mice (Fig 2F). Fructose water did not alter albumin leakage in both strains. Furthermore, Fructose water and a Western diet induced decreased plasma sodium (Fig 2G) and chloride levels (Fig 2H) compared to a control diet.
Figure 1. Metabolic Syndrome traits following fructose water or a Western-style diet in wild-type and Nlrp3KO mice. Average caloric intake (A) and weight gain (B) was calculated following 16 weeks of a Western-style diet or fructose water. Fold increase in weight gain in Wt or Nlrp3KO mice relative to its corresponding control diet (C). Weight of epididymal white adipose tissue (WAT) as a marker for abdominal obesity (D). Dyslipidemia was monitored with respect to plasma triglycerids (E). To evaluate insulin resistance, HOMA-IR (homeostatic model of insulin resistance) was calculated based on fasting blood glucose and insulin levels (F). Results are expressed as mean with SEM (n = 8 animals/group). Wild-type and Nlrp3KO mice are shown in respectively black and white bars. Control: control diet, control water; Fructose: control diet, fructose water, Western: western diet, control water. Statistics were performed using Kruskal Wallis with Dunn’s correction to assess dietary influence (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus corresponding Control group) and Mann-Whitney to assess influence of Nlrp3 deficiency (# = p < 0.05, ### = p < 0.01, #### = p < 0.001 versus corresponding Wt group).
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in wild-type mice within the narrow existing physiological range of plasma sodium and chloride. Nlrp3 deficiency partly restored plasma sodium and chloride levels in Western diet-fed mice. We next examined renal tissue sections and found that glomeruli, tubuli and capillaries showed a normal morphology in both control diet- and Fructose water-fed strains. In contrast, tubules of both wild-type and Nlrp3KO mice fed a Western diet showed extensive vacuolization (Fig 2J). Interestingly, quantification of vacuolated tubuli showed a decrease in Nlrp3KO versus wild-type mice fed a Western diet (Fig 2I).

**Figure 2.** Renal function and morphology of wild-type and Nlrp3KO mice fed fructose water or a Western-style diet. Renal gene expression of Nlrp3 (A) and Caspase-1 (B). Levels of urea (C), creatinine (D), uric acid (E) in plasma. Urinary albumin to creatinine ratio (ACR) (F). Plasma electrolyte levels of sodium (G) and chloride (H). Quantification of vacuolated tubuli per medium power field (MPF) (200x) scored by assessing 10 randomly selected non-overlapping MPFs in the renal cortex (I). PAS-D stained sections (400x) showing extensive vacuolization of proximal tubules (J). ND = not detectable. Results are expressed as mean with SEM (n = 8 animals/group). Wild-type and Nlrp3KO mice are shown in respectively black and white bars. Control: control diet, control water; Fructose: control diet, fructose water, Western: western diet, control water. Statistics were performed using Kruskal Wallis with Dunn’s correction to assess dietary influence (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus corresponding Control group) and Mann-Whitney to assess influence of Nlrp3 deficiency (# = p < 0.05, ## = p < 0.01, ### = p < 0.001 versus corresponding Wt group).
**Nlrp3 deficiency protects against a renal macrophage influx**

As inflammation is an important contributor to renal nephropathy, we next investigated renal leukocyte infiltration. Both Fructose water and a Western diet induced a Nlrp3-dependent macrophage influx in kidneys as seen by F4/80 immunohistochemistry (Fig 3A) and corresponding quantification (Fig 3B). No differences could be observed in the amount of granulocytes or T cells in all groups (data not shown). A Western diet induced MCP-1 in both wild-type and Nlrp3KO strains but did not correspond to macrophage influx (Fig 3C). Moreover, no difference in the inflammatory cytokines KC, TNF-α or IL-6 was detected (Supplemental Figure 2). We next investigated whether Nlrp3-dependent cytokine expression coincided with previously observed pathology. A Western diet induced IL-1β gene expression in wild-type mice but not in corresponding Nlrp3KO mice (Fig 3D). Protein expression showed no differential renal expression of IL-1β (Fig 3E). Immunoprecipitation of IL-1β in homogenates showed no discrete presence of active IL-1β in wild-type mice fed Fructose water or a Western diet (Fig 3F). In addition, neither ATP nor silica were able to induce IL-1β secretion in tubular epithelial cells in contrast to macrophages (data not shown). Since MCP-1 expression did not correlate with macrophage influx, we investigated intercellular adhesion molecule-1 (ICAM-1), a regulator of mononuclear cell infiltration in the renal interstitium24,25. ICAM-1 protein expression was strongly enhanced in the tubulointerstitial compartment of wild-type mice fed Fructose water or a Western diet compared to a control diet (Fig 3G). Both Fructose water and a Western diet upregulated ICAM-1 expression in the tubulointerstitial compartment which was prevented by Nlrp3 deficiency (Fig 3H). More specifically, endothelial ICAM-1 expression was increased in groups experiencing an increased macrophage influx (Supplemental Figure 3), i.e. wild-type mice fed Fructose water or a Western diet.

**Nlrp3 deficiency protects against MetSyn-associated renal fibrosis**

Renal fibrosis is a hallmark of MetSyn-driven renal pathology. We therefore investigated the role of Nlrp3 in the development of fibrosis by examining gene expression of three fibrotic markers in kidney. A significant difference was observed in connective tissue growth factor (CTGF) (Fig 4A), collagen type 1 (Fig 4B) and fibronectin (Fig 4C) gene expression in wild-type mice versus Nlrp3KO mice fed a Western diet. In line with increased collagen type 1 gene expression, we observed increased collagen type 1 protein expression by
Figure 3. Renal inflammation in wild-type and Nlrp3KO mice fed fructose water or a Western-style diet. Macrophage F4/80 immunohistochemistry of corticomedullary region (200x) (A). Quantification of F4/80+ pixels in whole kidney sections (B). Renal MCP-1 protein expression normalized for total protein content (C). Renal gene expression of interleukin-1β (IL-1β) as determined by quantitative PCR, normalized for gene expression of TATA-box binding protein (TBP) (D). Protein expression as determined by ELISA of IL-1β normalized for total protein content (E). Immunoprecipitation and Western blot of IL-1β including a negative control (-) and positive control (+) control for immunoprecipitation and in addition 50 pg of IL-1β (IL-1β) as a positive control for Western blotting (F). Quantification of ICAM-1+ pixels in whole kidney sections (G). ICAM-1 immunohistochemistry of corticomedullary region (200x) (H). Results are expressed as mean with SEM (n = 8 animals/group). Wild-type and Nlrp3KO mice are shown in respectively black and white bars. Control: control diet, control water; Fructose: control diet, fructose water; Western: western diet, control water. Statistics were performed using Kruskal Wallis with Dunn’s correction to assess dietary influence (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus corresponding Control group) and Mann-Whitney to assess influence of Nlrp3 deficiency (# = p < 0.05, ## = p < 0.01, ### = p < 0.001 versus corresponding Wt group).
immunohistochemistry (Fig 4E). Collagen type 1 accumulation was present in the tubulointerstitium and most pronounced peri-glomerular. Nlrp3 deficiency prevented Western diet-induced peri-glomerular collagen type 1 accumulation. Quantification of peri-glomerular collagen type 1 accumulation revealed a Western diet-induced Nlrp3-dependent effect (Fig 4D). Fructose water did not induce any of the three fibrotic markers (Fig 4D).

**Nlrp3 deficiency protects against MetSyn-associated renal steatosis**

Since we observed extensive tubular vacuolization in wild-type mice fed a Western diet, we speculated that vacuolization may underlie related nephropathy. As tubular vacuoles exhibited the morphology of lipid-loaded vesicles, we investigated renal lipid content. Non-esterified fatty acids (Fig 5A) and triglyceride levels (Fig 5B) remained however unchanged. In contrast, both cholesterol (Fig 5C) and

![Figure 4](image-url)

**Figure 4.** Renal fibrosis in wild-type mice and Nlrp3KO mice fed a Western-style diet or fructose water. Renal gene expression of fibrotic markers as determined by quantitative PCR: connective tissue growth factor (CTGF) (A), collagen type 1 (B) and fibronectin (C) normalized for gene expression of TBP. Quantification of glomeruli with >50% peri-glomerular collagen type 1 accumulation per medium power field (MPF) (200x) scored by assessing 10 randomly chosen renal cortical MPFs (D). Collagen type 1 immunohisto-chemistry of cortical region (400x) (E). Results are expressed as mean with SEM (n = 8 animals/group). Wild-type and Nlrp3KO mice are shown in respectively black and white bars. Control: control diet, control water; Fructose: control diet, fructose water, Western: western diet, control water. Statistics were performed using Kruskal Wallis with Dunn’s correction to assess dietary influence (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus corresponding Control group) and Mann-Whitney to assess influence of Nlrp3 deficiency (# = p < 0.05, ## = p < 0.01, ### = p < 0.001 versus corresponding Wt group).
Figure 5. Renal lipid homeostasis in wild-type and Nlrp3KO mice fed fructose water or a Western-style diet. Non-esterified fatty acids (NEFA) (A), triglycerids (B), total cholesterol (C) and phospholipids (D), measured in kidney homogenates and normalized for protein content. Correlation between the average amount of vacuoles per medium power field (200x) in PAS-D sections and the amount of total cholesterol (E) or phospholipids (F) present in kidney homogenates. Results are expressed as mean with SEM (n = 8 animals/group). Wild-type and Nlrp3KO mice are shown in respectively black and white bars. Control: control diet, control water; Fructose: control diet, fructose water, Western: western diet, control water. Statistics were performed using Kruskal Wallis with Dunn’s correction to assess dietary influence (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 versus corresponding Control group) and Mann-Whitney to assess influence of Nlrp3 deficiency (# = p < 0.05, ## = p < 0.01, ### = p < 0.001 versus corresponding Wt group) (A-D) and Spearman (E,F).
phospholipid (Fig 5D) content was increased in wild-type mice fed a Western diet versus a control diet which was prevented by Nlrp3 deficiency. When correlating the amount of vacuoles present in PAS-D sections with renal cholesterol (Fig 5E) or phospholipid (Fig 5F) levels, we found a highly significant correlation for both lipids. These findings were compatible with the observation that tubular vacuoles were positive for the lipophilic compound Nile Red (Fig 6A-D). Observed orange fluorescence indicated the presence of phospholipids rather than cholesterol since cholesterol will experience a blue shift towards yellow-gold fluorescence (26). Spectral imaging revealed increased numbers of Nile Red-positive vacuoles in wild-type mice but not in Nlrp3KO mice fed a Western diet compared to corresponding control diet-fed mice (Fig 6E-H). Glycogen deposits and cholesterol crystals were not observed using PAS-staining and electron microscopy or polarizing light microscopy, respectively (data not shown).
Renal SREBP-2 and LDLR expression associates with cholesterol accumulation

As cellular cholesterol accumulation is cytotoxic and stimulates an adaptive phospholipid biosynthesis response (27–29), we next focused on the mechanism by which Nlrp3 deficiency prevents renal cholesterol accumulation by analysing genes of the cholesterol biosynthesis pathway. Expression of cholesterol import-associated genes CD36, scavenger receptor B1 and the cholesterol synthesis
gene HMG CoA Reductase remained unaltered (data not shown). In addition, expression of the cholesterol export-associated gene ABCA-1 was similar between Wt and Nlrp3KO mice fed a Western diet (data not shown). In contrast, import-associated low density lipoprotein receptor (LDLR) was highly increased in Fructose water- or Western diet-fed wild-type mice versus a control diet but absent in corresponding Nlrp3KO mice (Fig 7A). In line with LDLR expression, gene expression of the master transcriptional regulator of cholesterol synthesis sterol regulatory element binding protein-2 (SREBP-2) (30) is enhanced after Fructose water and a Western diet (Fig 7B). This diet-induced response was significantly prevented in kidneys of Nlrp3KO mice. Similarly, SREBP-1c was also upregulated in wild-type mice fed Fructose water or a Western diet which was prevented in Nlrp3KO mice fed a Western diet (Fig 7C). Finally, we observed a shift in total body cholesterol distribution between wild-type and Nlrp3KO mice fed a Western diet (Fig 7D). Cholesterol accumulated in kidneys of wild-type mice fed a Western diet (Fig 5C) whereas corresponding Nlrp3KO mice instead displayed hypercholesterolemia (Fig 7C), in line with increased renal LDLR expression in wild-type mice (Fig 7A).

Discussion

MetSyn is a growing worldwide health problem with genetic and environmental causes, which is related to the development of renal disease and a state of chronic, low-grade inflammation. The potential link between MetSyn, inflammation, and renal disease is largely unknown. Here, we investigated the role of Nlrp3 on the development of MetSyn and associated renal disease and inflammation using two different diets. We show that Fructose water induces Nlrp3-dependent nephropathy without development of MetSyn. A Western diet induced MetSyn to a similar extent in wild-type and Nlrp3KO mice. Despite this, subsequent renal injury was prevented by Nlrp3 deficiency as characterized by the absence of renal inflammation, fibrosis, micro-albuminuria and hyperuricemia. MetSyn-associated pathology was accompanied by a profound Nlrp3-dependent renal cholesterol and phospholipid accumulation.

Fructose is increasingly consumed in modern Western-style diets (e.g. beverages) and has been associated with the high prevalence of the metabolic syndrome in humans (31,32). However, current data on the role of fructose on MetSyn are inconclusive and somewhat contradictory. Here we show that the chronic
consumption of 15% fructose-rich water, the highest amount of fructose in U.S. brands of fructose-sweetened soft drinks, does not induce MetSyn. In spite of this, renal inflammation and plasma sodium and chloride levels were negatively affected after fructose consumption, a response that was prevented in mice lacking Nlrp3. No effect of fructose consumption and the role of Nlrp3 herein were found on the development of renal fibrosis, and steatosis. Next to fructose, a Western diet rich in cholesterol is known to be involved in MetSyn development. In line we found increased adiposity and insulin resistance after a Western diet in both wild-type and Nlrp3KO mice. Given that the relative weight gain of wild-type and Nlrp3KO mice was equal after a Western diet despite an increased caloric intake in Nlrp3KO mice suggests that 1) Nlrp3 deficiency increases caloric intake from a Western diet and 2) Nlrp3 deficiency protects against Western diet-induced relative weight gain. These data are in concordance with literature showing that Nlrp3 deficiency protects against obesity after a high-fat diet (17–19) although the outcome after a Western diet was much less pronounced. The effect we found of Nlrp3 deficiency on caloric intake mimicks the observation that deficiency of Caspase-1, part of the Nlrp3 inflammasome, interfered with resistin secretion, a satiety signal (17). Of particular interest is the observation that Nlrp3 deficiency prevented hyperuricemia, micro-albuminuria and a drop in plasma sodium and chloride after a Western diet. Decreasing plasma sodium and chloride levels together with decreased plasma urea in Western diet-fed mice suggests that more water is being retained. Increased co-peptin levels, a marker for antidiuretic hormone (ADH), have been observed in patients with micro-albuminuria and MetSyn (33) which stimulates water retention. To explore the mechanism by which Nlrp3 deficiency protects against diet-induced nephropathy we analysed renal inflammation, fibrosis and steatosis. The Nlrp3 inflammasome-dependent cytokine IL-1β was not upregulated and no discrete presence of active IL-1β was observed indicating an inflammasome-independent phenotype similar to observations made by Shigeoka et al.(34). However, we did observe a Nlrp3-dependent renal macrophage influx after Fructose water or a Western diet. This agrees with previous studies showing that macrophage depletion protects against loss of kidney function and fibrosis during experimental renal disease (35,36). Furthermore, macrophage accumulation was linked to the development of diabetic nephropathy (37). Renal KC and IL-6 levels were increased in Nlrp3KO groups whereas no inflammatory cell influx could be observed in
these groups. We speculate that this discordance is derived from the fact that Nlrp3KO mice show increased weight gain which associates with an increased state of systemic inflammation. Therefore, increased renal chemokines levels are rather a reflection of increased systemic inflammation rather than increased renal inflammation. In addition, locally regulated proteins such as adhesion molecules play a role in inflammatory cell influx. ICAM-1 expression is crucial for leukocyte-endothelium adhesion in the renal interstitium (24,25). Interestingly, Nlrp3 deficiency strongly prevented tubulointerstitial and endothelial ICAM-1 upregulation by Fructose water or Western diet, possibly limiting macrophage influx. The prevention of ICAM-1 upregulation in the absence of Nlrp3 can be explained by the lower renal cholesterol levels or fructose intake we found in respectively Nlrp3KO mice fed a Western diet or Fructose water. Indeed, endothelial cells upregulate ICAM-1 when stimulated with either cholesterol (38) or fructose (39).

In addition, we observed enhanced renal fibrosis after consumption of a Western diet which was not observed after drinking fructose water. The former observation is in line with data showing that a high cholesterol diet induces liver fibrosis (40,41). The induction of a renal fibrogenic and pro-inflammatory response in the kidney after feeding a Western diet is associated with a local CTGF induction. Indeed, CTGF is an important pro-fibrotic factor in kidney (42,43) that can promote leukocyte infiltration in the renal interstitium (44). We found that Nlrp3KO mice were protected against Western diet-induced renal fibrosis possibly because the upregulation of local CTGF is prevented. The reason for this remains elusive but could involve differences in cellular stress (reflected by absent hyperuricemia, and a better preserved plasma sodium and chloride level) that is known to induce CTGF (45).

Based on renal inflammation, fibrosis, morphology and functional parameters, we conclude that a Western diet is much more detrimental to the kidney compared to Fructose water when administered in physiological amounts. Therefore, we focused on understanding the mechanism by which Nlrp3 deficiency protects against Western diet-induced nephropathy by analyzing renal steatosis. We found significant increases in renal phospholipid and cholesterol levels and intratubular lipid droplets after a Western diet, which was prevented when Nlrp3 was absent. Following the lipid nephrotoxicity hypothesis postulated by John Moorhead in 1982 (46) and updated in 2009 (47), we speculated that this cholesterol accumulation is able to cause tubular dysfunction. Tubuli are
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responsible for resorption of electrolytes within a narrow range in order to induce an osmotic gradient and control water retention as well as for the excretion of uric acid into urine. Consistent with this notion, we found hyperuricemia, reduced plasma sodium and chloride retention and enhanced renal cholesterol levels after a Western diet. Responses that were all prevented in Nlrp3KO mice after consuming a Western diet. Besides being a consequence of renal disease, hyperuricemia in wild-type mice can also be the cause of MetSyn-associated renal diseases (48).

Interestingly, we demonstrate that cholesterol and phospholipid accumulation in kidneys coincides with increased renal SREBP-2, LDLR and SREBP-1c expression in wild-type mice whereas Nlrp3 deficiency prevented this response and confined cholesterol accumulation to plasma. Nlrp3-dependent renal cholesterol accumulation can be explained by a study of Gurcel and colleagues that showed that the Nlrp3 inflammasome is able to interfere with cholesterol homeostasis through modulation of SREBP-2 in a bacterial infection model (49). In line with our results SREBP-2 can activate the LDLR gene and induce cholesterol accumulation. SREBP-1c is activated by insulin and oxysterols and activation leads to SREBP-1c transcription and phospholipid synthesis (30). We speculate that cholesterol accumulation drives oxysterol-mediated SREBP-1c activation and downstream phospholipid accumulation. However, recent literature showed that SREBP-2 is able to activate Nlrp3 (51) suggesting a role for SREBP-2 upstream of Nlrp3 without the need for Caspase-1 activation. The role of Caspase-1 in our study is unclear and deserves additional attention.

Upregulated LDLR expression in wild-type fed Fructose water mice did not induce cholesterol accumulation which suggests that LDLR upregulation is only deleterious combined with a cholesterol-containing diet. Nlrp3 can be activated by uric acid crystals (52), cholesterol crystals (53), 7-ketocholesterol (54), ceramide (19) and saturated fatty acid palmitate (55), all metabolites of fructose or a Western diet. Although we found higher renal cholesterol levels and hyperuricemia in wild-type mice after both diets, the presence of an obvious Nlrp3 activator, such as cholesterol and uric acid crystals could not be observed using electron and polarized light microscopy. Further studies will be necessary to investigate the presence and role of other Nlrp3 activators in our model such as the oxysterol 7-ketocholesterol and ceramide (19,54,55) that can be formed respectively during cholesterol catabolism, or degradation of diet-derived fatty acids.
CHAPTER 2

Here, we show that Nlrp3 is a link between a Western diet or fructose consumption and the development of renal disease. We speculate that Nlrp3 drives diet-induced nephropathy through macrophage infiltration and SREBF2- and LDLR-mediated cholesterol accumulation. Our study reveals a novel role for Nlrp3 in regulating cholesterol accumulation, upstream of its current role as a detector of cholesterol accumulation during atherosclerosis. More research is needed to investigate the therapeutic potential of inhibiting Nlrp3 in cholesterol-associated disease.

**Supplemental figures**

Supplemental figures and tables can be found online.
References


CHAPTER 2


