Tampere University

VENLA KURRA

Uric acid, Cardiovascular Function, and the Kidney

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ACADEMIC DISSERTATION
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Dedicated to those who venture their own path

"The only true voyage of discovery… would be not to visit strange lands, but to possess other eyes, to behold the universe through the eyes of another, of a hundred others, to behold the hundred universes that each of them beholds, that each of them is."

Marcel Proust

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ABSTRACT

Uric acid (UA) is the end-product of purine metabolism in human. The most common clinical manifestation of elevated circulating UA level, i.e. hyperuricemia, is gout, an inflammatory arthritis typically manifesting in the first metatarsophalangeal joint. Epidemiological studies have rather unambiguously indicated that the prevalence of hyperuricemia is increasing worldwide. This increase has co-occurred with lifestyle westernization and higher incidence of chronic kidney disease (CKD), and cardiovascular morbidities such as hypertension, type 2 diabetes, and atherosclerosis, suggesting a possible role of UA in the development of these diseases. It is still not clear, however, whether UA itself plays a role in these conditions, or is secondary to reduced renal UA excretion and oxidative stress, both elevating circulating UA levels. The ability of UA to provide protection against oxidant damage in certain neurological conditions challenges the view of UA being harmful contributor in disease pathophysiology.

To investigate the role of UA in cardiovascular and renal diseases, an experimental model of dietary 2% oxonic acid (inhibitor of uric acid degrading uricase-enzyme)-induced hyperuricemia has been developed. The model rather closely simulates the hyperuricemic state in humans, in whom a mutation early in the evolution has resulted in higher UA levels than in most other mammals. Previous experimental studies with rather short durations and heterogenic study protocols have suggested that UA contributes to the progression of cardiovascular and renal diseases by causing intrarenal inflammation, oxidative stress, and depletion of endothelium-derived vasodilator nitric oxide (NO). Synergistically, these changes have been suggested to cause glomerular vascular disease, hypertension, and ultimately renal fibrosis. The contribution of UA to the systemic vascular function, morphology, and renal histology in relation to the prevailing oxidant status has not been previously elucidated.

Reduced compliance of the large arteries, as indicated by increased pulse wave velocity (PWV), is considered as an independent indicator for higher cardiovascular risk. In some clinical studies, higher UA levels have been independently associated

with increased PWV, while other studies have not supported this view. Thus, the knowledge over the role of UA in human hemodynamics is largely unknown.

The experimental part of the study (studies I, II, III) examined the cardiovascular and renal effects of nine week-long oxonic acid (Oxo) induced hyperuricemia in healthy (Sham) and surgically 5/6 nephrectomized (NX) rats (n=12/group). The focus was to investigate the mesenteric and carotid artery functional responses, small mesenteric artery morphology, cardiac volume load, systemic and renal oxidative stress, and kidney markers of fibrosis and inflammation. The vascular studies were performed *in vitro* with isolated arterial rings by recording the changes in small artery tone in response to different pharmacological vasoactive agents by using a myograph. Cardiac load was assessed by measuring cardiac natriuretic peptide mRNA content, while systemic oxidative stress markers and antioxidant status were evaluated *in vivo* by measuring urinary excretion of 8-isoprostaglandin $F_{2\alpha}$ and 11-epi-prostaglandin $F_{2\alpha}$ and plasma total peroxyl radical-trapping capacity (TRAP). Indices of renal damage (histology, proteinuria, mast cell count), inflammation (cyclooxygenase-2, COX-2), and oxidative stress (heme oxygenase-1, HO-1) were evaluated microscopically, immunohistochemically, and by using Western blotting and real time quantitative PCR (RT-PCR), as appropriate. The clinical study (study IV) examined non-invasively by using whole-body impedance cardiography and radial tonometric pulse wave analysis the association of plasma UA concentration with several hemodynamic variables, including PWV. The study participants consisted of 606 normotensive or never-medicated hypertensive asymptomatic individuals with plasma UA levels predominantly within the normal range.

The results from the experimental study replicated several characteristics of moderate CKD: mild hypertension and cardiac load, impaired endothelium-mediated vasorelaxation in the mesenteric and carotid artery, hypertrophic remodeling of the small mesenteric artery, renal fibrosis, proteinuria, and increased oxidative stress. In experimental CKD, hyperuricemic milieu improved NO-mediated carotid artery vasorelaxation, but in parallel endothelium-independent vasorelaxation mediated via smooth muscle cell hyperpolarization in the main branch of the mesenteric artery was impaired. Congruently, hyperuricemic remnant kidney rats displayed improved kidney morphology and reduced markers of inflammation. Systemic and renal markers of oxidative stress were reduced, and plasma TRAP increased in hyperuricemia, but UA did not significantly influence blood pressure, cardiac load, or small mesenteric artery morphology. In the clinical

study, plasma UA concentration was independently associated with large arterial stiffness in both sexes, but not with blood pressure or any other hemodynamic variable including cardiac output, systemic vascular resistance, or wave reflection.

The present series of experimental studies adds further knowledge to the mechanisms via which UA mediates its effects in the cardiovascular system and in the kidney. In experimental CKD, hyperuricemia associated with improved renal morphology and NO-mediated carotid artery vasorelaxation, in association with more favorable oxidant/antioxidant status both *in vivo* and in the kidney. The results from the clinical study support the view that in normotensive and never-medicated hypertensive individuals, the association of higher UA levels with increased cardiovascular risk might be mediated via reduced compliance of the large arteries. Collectively, these results demonstrate the dual actions of UA in different species and in physiological versus *in vitro* milieus. Prospective longitudinal studies exploring the hemodynamic effects of hyperuricemia are warranted in the future.

TIIVISTELMÄ

Uraatti on ihmisillä puriiniaineenvaihdunnan lopputuote, joka pääsääntöisesti poistuu elimistöstä erittymällä munuaisten kautta virtsaan. Uraatin kohonnut pitoisuus veressä, hyperurikemia, on tavallinen löydös paitsi kihtiä, myös sydän- ja verenkiertoelimistön sairauksia sekä kroonista munuaisten vajaatoimintaa sairastavilla potilailla. Näillä potilailla hyperurikemia on seurausta sairauksiin yleisesti liittyvästä oksidatiivisen kuormituksen lisääntymisestä sekä munuaisten erityskyvyn heikkenemisestä. Hyperurikemia liitetään usein sairauksien huonompaan ennusteeseen, mutta tunnettujen antioksidanttivaikutustensa kautta uraatti voi myös vähentää elimistön oksidatiivista kuormitusta. Uraatin rooli ja vaikutusmekanismit verenkiertoelimistön sairauksissa sekä kroonisessa munuaisten vajaatoiminnassa ovat edelleen kiisteltyjä.

Ihmisen uraattipitoisuudet ovat korkeammat kuin muilla nisäkkäillä johtuen ihmisillä aikoinaan tapahtuneesta uraattia hajottavan urikaasi-entsyymin mutaatiosta. Uraatin vaikutusten tutkimista varten kehitetyssä mallissa ihmisen hyperurikemiaa jäljittelevä hyperurikemia saadaan aikaiseksi antamalla ravinnon mukana koe-eläiminä käytetyille rotille urikaasin estäjää, 2% oksonihappoa. Aikaisemmissa valtaosin aika lyhyissä ja koeasetelmiltaan vaihtelevissa kokeellisissa tutkimuksissa näin aiheutetun hyperurikemian on esitetty aiheuttavan munuaisvaurioita lisääntyneen oksidatiivisen kuormituksen, tulehduksen sekä munuaisverisuonten toiminnallisten ja rakenteellisten muutosten kautta. Lopulta nämä muutokset johtavat verenpainetason kohoamiseen, edelleen kiihdyttäen munuaissairauden etenemistä. Aikaisemmat kokeelliset tutkimukset ovat keskittyneet lähinnä uraatin munuaisvaikutuksiin, mutta hyperurikemian yhteyttä muiden verisuonten toimintaan, rakenteeseen tai oksidatiivisen kuormitukseen ei ole aikaisemmin kattavasti tutkittu.

Väitöskirjatyön kokeellisessa osuudessa (osatyöt I, II, III) selvitettiin kohtalaisesti kohonneen uraattipitoisuuden vaikutusta kaula- ja suolilievevaltimon toimintaan ja rakenteeseen, sydämen kuormitukseen, munuaishistologiaan ja -tulehdukseen sekä elimistön oksidatiiviseen kuormitukseen terveillä ja kroonista munuaisten

vajaatoimintaa sairastavilla rotilla (neljä ryhmää, n=12/ryhmä). Hyperurikemia aiheutettiin antamalla osalle koe-eläimistä ravinnon mukana 2% oksonihappoa yhdeksän viikon ajan ja munuaisten vajaatoiminta saatiin aikaiseksi poistamalla osalta rotista kirurgisesti 5/6 munuaiskudoksesta (NX-toimenpide, kolme viikkoa ennen oksonihappodieettiä). Verisuonten rakennetta sekä supistus- ja relaksaatiovastetta eri tavalla verisuonten toimintaan vaikuttaviin aineisiin tutkittiin *in vitro* verisuonen supistustilaa mittaavalla myografialaitteella. Sydämen kuormitusta arvioitiin määrittämällä sydänlihaksen natriureettisten peptidien mRNA-pitoisuuksia. Munuaisvauriota arvioitiin histologisesti, virtsan proteiinierityksen mittauksilla sekä määrittämällä erilaisia munuaisvaurioon ja oksidatiiviseen kuormituksen viittaavia tekijöitä (mm. mast-solut, hemi-oksygenaasi-1-entsyymi, syklo-oksigenaasi-2 ja kollageeni-1). Oksidatiivista kuormitusta ja antioksidanttikapasiteettia arvioitiin myös *in vivo* määrittämällä näistä kertovia merkkiaineita verestä ja virtsasta (TRAP, 8-isoprostaglandiini $F_{2\alpha}$ ja 11-epi-prostaglandin $F_{2\alpha}$).

Käytetty koe-eläinmalli toimi odotetusti ja tutkimuksessa havaittiin useita aikaisemmin kuvattuja munuaisten vajaatoimintaan liittyviä löydöksiä, kuten lievä verenpaineen nousu, verisuonten heikentynyt relaksaatio, vastusverisuonten seinämän paksuuntuminen, sydämen kuormitus, proteinuria, lisääntynyt oksidatiivinen kuormitus sekä munuaisten histologinen vaurio. Munuaisten vajaatoiminnassa hyperurikemia paransi kaulavaltimon endoteelivälitteistä (typpioksidivälitteistä) relaksaatiota, kun taas suolilievevaltimossa hyperurikemia heikensi sileälihaksen hyperpolarisaation kautta tapahtuvaa vasorelaksaatiota. Munuaisten vajaatoiminnassa kokeellinen hyperurikemia lievensi histologista munuaisvauriota ja tämä liittyi vähentyneeseen systeemiseen sekä munuaisten oksidatiiviseen kuormitukseen. Havaitut suotuisat vaikutukset munuaisten vajaatoimintaa sairastavilla rotilla selittyivät todennäköisesti hyperurikemiaan liittyneellä plasman kokonaisantioksidanttikapasiteetin lisääntymisellä sekä elimistön vähentyneellä oksidatiivisella kuormituksella. Hyperurikemialla ei ollut merkittävää vaikutusta verenpaineeseen, verisuonten supistumiseen, pienten vastusverisuonten rakenteeseen tai sydämen kuormitukseen.

Suurten valtimoiden jäykistyminen on tunnettu sydän- ja verisuonisairauksien riskitekijä ja kohonnut uraattipitoisuus on yhdistetty itsenäisenä riskitekijänä valtimojäykkyyttä kuvaavaan lisääntyneeseen pulssiaallon etenemisnopeuteen (PWV). Tutkimuksen kliinisessä osatyössä (osatyö IV) tutkittiin kajoamattomin menetelmin (impedanssikardiografia, tonometrinen radialispaineiden mittaus)

pääsääntöisesti viiterajoissa olevien uraattitasojen yhteyttä erilaisiin hemodynaamisiin muuttujiin 606:lla sydän- ja verisuonisairauksien suhteen terveellä normaaliverenpaineisella tai ei-lääkityillä hypertensiivisellä koehenkilöllä. Tutkimuksessa havaittiin, että plasman uraattipitoisuus on PWV:tä ja siten myös valtimojäykkyyttä selittävä tekijä, mutta muihin mitattuihin hemodynaamisiin suureisiin, mukaan lukien verenpaine verenkierron vastus, minuuttitilavuus ja paineheijasteet, uraattitasoilla ei ollut yhteyttä.

Nyt tehty koe-eläintutkimus osoittaa, että kokeellisen hyperurikemian vaikutukset verenkiertoelimistössä ja munuaisissa eroavat terveillä sekä munuaisten vajaatoimintaa sairastavilla rotilla, ja vaikutukset verisuonten toimintaan ovat erilaisia eri verisuonissa. Tässä koe-eläinmallissa hyperurikemialla ei pääsääntöisesti havaittu olevan haitallisia vaikutuksia. Suotuisat verisuoni- ja munuaislöydökset munuaisten vajaatoimintaa sairastavilla rotilla voisivat ainakin osittain selittyä hyperurikemiaan liittyneellä oksidatiivisen kuormituksen vähentymisellä. Kliinisen työn tulokset osoittavat, että korkeampi, vaikkakin viiterajoissa oleva, veren uraattipitoisuus liittyy lisääntyneeseen valtimojäykkyyteen, mikä on havaittavissa jo ennen kliinisesti todettavaa verenpaineen nousua tai muita sydän- ja verisuonisairauksien merkkejä. Tämä yhteys saattaa osaltaan selittää korkeampiin uraattipitoisuuksiin liittyvää lisääntynyttä sairastavuutta sydän- ja verenkiertoelimistön sairauksiin. Tältä osin tarvitaan kuitenkin vielä seurantatutkimuksia.

CONTENTS

ABBREVIATIONS

8-iso-$PGF_{2\alpha}$	8-isoprostaglandin $F_{2\alpha}$
11-epi-$PGF_{2\alpha}$	11-epi-prostaglandin $F_{2\alpha}$
Ach	Acetylcholine
ACR	American College of Rheumatology
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ADP	Adenosine triphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BK_{Ca}	Large-conductance Ca^{2+}-activated K^{+}-channels
ß-MHC	ß–myosin heavy chain
BMI	Body mass index
BNP	B-type natriuretic peptide
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CI	Cardiac index
CKD	Chronic kidney disease
CO	Cardiac output
COX-2	Cyclooxygenase-2
CRI	Chronic renal insufficiency
CRP	C-reactive protein
CTGF	Connective tissue growth factor
DNA	Deoxyribonucleic acid
EDHF	Endothelium-derived hyperpolarizing factor
EET	Epoxyeicosatrienoic acid
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease

EULAR	European League Against Rheumatism
GFR	Glomerular filtration rate
GLUT	Glucose transporter
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HDL	High-density lipoprotein
HETE	Hydroxyeicosatetraenoic acid
HO-1	Heme oxygenase-1
HPRT1	Hypoxanthine-guanine phosphoribosyl transferase
HR	Heart rate
LDL	Low-density lipoprotein
L-NAME	N^G-nitro-L-arginine methyl ester
LVH	Left ventricle hypertrophy
mRNA	Messenger ribonucleic acid
ß-MHC	Beta-myosin heavy chain
NA	Noradrenaline
NAD	Nicotinamide adenine dinucleotide
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
NX	5/6 nephrectomy
OAT	Organic anion transporter
Oxo	2% oxonic acid diet
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
PGI_2	Prostacyclin
PWV	Pulse wave velocity
QUICKI	Quantitative insulin sensitivity check index
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time quantitative polymerase chain reaction
SEM	Standard error of the mean
SI	Stroke index
SVR	Systemic vascular resistance
SVRI	Systemic vascular resistance index

SMA	Smooth muscle cell alpha-actin
SLGT2	Sodium-glucose co-transporter-2
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SUA	Serum uric acid
SV	Stroke volume
TGF-β	Transforming growth factor-β
TRAP	Total peroxyl radical-trapping capacity
UA	Uric acid
URAT	Urate anion transporter
VSMC	Vascular smooth muscle cell
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

ORIGINAL PUBLICATIONS

Publication I Venla Kurra, Arttu Eräranta, Pasi Jolma, Tuija I. Vehmas, Asko Riutta, Eeva Moilanen, Anna Tahvanainen, Jarkko Kalliovalkama, Onni Niemelä, Juhani Myllymäki, Jukka Mustonen and Ilkka Pörsti: Hyperuricemia, Oxidative stress, and carotid artery tone in experimental renal insufficiency. American Journal of Hypertension 2009; 22: 964-970.

Publication II Venla Kurra, Tuija Vehmas, Arttu Eräranta, Jarkko Jokihaara, Päivi Pirttiniemi, Heikki Ruskoaho, Heikki Tokola, Onni Niemelä, Jukka Mustonen and Ilkka Pörsti; Effects of oxonic acid-induced hyperuricemia on mesenteric artery tone and cardiac load in experimental renal insufficiency. BMC Nephrology 2015; 16: 35.

Publication III Venla Kurra, Arttu Eräranta, Timo Paavonen, Teemu Honkanen, Juhani Myllymäki, Asko Riutta, Ilkka Tikkanen, Päivi Lakkisto, Jukka Mustonen and Ilkka Pörsti. Moderate hyperuricemia ameliorated kidney damage in a low-renin model of experimental renal insufficiency. Submitted manuscript.

Publication IV Humam Hamid, Venla Kurra, Manoj Kumar Choudhary, Heidi Bouquin, Onni Niemelä, Mika A.P. Kähönen, Jukka T. Mustonen, Ilkka H. Pörsti and Jenni K. Koskela. Plasma uric acid is related to large arterial stiffness but not to other hemodynamic variables: a study in 606 normotensive and never-medicated hypertensive subjects. BMC Cardiovascular Disorders 2021; 21: 257.

THE AUTHOR´S CONTRIBUTION

Publications I-III	Venla Kurra, together with the other group members, had a crucial role in planning and performing the animal experiments (including the measurements during the follow-up, sample collection and functional vascular experiments). Venla Kurra had an essential contribution to data analyses and in drafting and reviewing the manuscript.
Publication IV	This study is a part of the hemodynamic study group work, in which Venla Kurra has been participating. Venla Kurra's contribution consists of data collection, examining the study participants and interpreting the study results. In this publication Venla Kurra contributed to reviewing of the manuscript.

1 URIC ACID AND HYPERURICEMIA

Uric acid is the final break down product of amino acids and purine nucleic acids (adenine and guanine) in humans. Nucleic acids are derived either endogenously from the cellular turnover of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), guanosine triphosphate (GTP) or exogenously mainly via purine rich animal proteins. Approximately two-thirds of the total body UA is derived endogenously, and the remaining one-third via digestion. Uric acid is synthesized mainly in the liver but also in other tissues such as the kidney, vascular endothelium and striated muscle, while the kidneys are the predominant pathway for UA excretion (Chaudhary et al., 2013; Maiuolo et al., 2016).

In the biomedical literature, the term uric acid generally refers to undissociated diprotic form of UA. In the plasma (at physiologic pH), UA exists mostly as dissolved sodium urate acid, the salt of UA. To refer to the total pool of circulating UA, the terms UA and urate are often used interchangeably, as their ratio remains practically constant.

1.1 Metabolism of uric acid

The synthesis of UA is catalyzed sequentially by several enzymes, the rate-limiting enzyme being xanthine oxidoreductase (XOR) (C. Chen et al., 2016; Maiuolo et al., 2016) (Figure 1). This enzyme is responsible for the two terminal reactions in UA generation: oxidation of hypoxanthine to xanthine and xanthine to UA. The activity levels of XOR in human varies in different tissues, the highest levels observed in the liver, intestine and endothelium and the lowest in the brain, skeletal muscle, and the heart (Chaudhary et al., 2013). Xanthine oxidoreductase can attain two alternative enzymatic isoforms, namely xanthine oxidase (XO) and xanthine dehydrogenase (XDH), both generating UA. The first one uses molecular oxygen as an electron acceptor and releases UA, hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS), most importantly superoxide anion (O_2^-). The reaction catalyzed by XDH

utilizes nicotinamide adenine dinucleotide (NAD^+) as a cofactor and generates the reduced form of NAD (NADH) and hydrogen ion (H^+) (C. Chen et al., 2016; Maiuolo et al., 2016). Xanthine dehydrogenase and XO can be interconverted via proteolytic action or oxidation of sulfhydryl residues (Nishino et al., 2008). In physiologic conditions, XDH is constitutively expressed, while in tissue ischemia XO is the predominant pathway generating both UA and free radicals (Berry & Hare, 2004). The synthesis of UA is therefore closely coupled to a range of pathological conditions with enhanced XO activity and free radical generation, and UA can therefore be regarded as an index for oxidative stress (Packer, 2020). Whether UA *per se* plays a role in these states, however, is still debated. Recently it was reported that plasma XOR activity is related to higher blood pressure (BP) through ROS, but not UA generation (Yoshida et al., 2020).

In humans, on average two-thirds of the circulating UA is excreted by the kidneys, where UA is almost totally filtered through the glomeruli (Hyndman et al., 2016). Renal UA transport occurs mainly in the proximal tubule, where 95-95% of the filtered UA is reabsorbed. Though reabsorption has the predominant role in renal UA transport, there is a secretory component as well, with a relatively larger importance in some other species (for example reptiles and birds) than human or rat (So & Thorens, 2010). Post-secretory reabsorption more distally in the proximal tubule also contributes to renal UA processing (Maiuolo et al., 2016). Renal urate handling is regulated by urate transporters which exchange intracellular organic anions for UA. The most important urate reabsorption transporter located in the apical membrane of the proximal tubule is a member of organic anion transporter (OAT) family, urate anion transporter 1 (URAT1). Other reabsorption transporters include OAT4 (organic anion transporter 4), OAT10 and glucose transporter 9 (GLUT9). Urate secretion transporters include many other members of the OAT family and various other transporters, many of them still not fully characterized (Bobulescu & Moe, 2012; Hyndman et al., 2016; Sorensen & Levinson, 1975).

The remaining one-third of circulating UA is predominantly excreted via the gastrointestinal route, where it is metabolized by gut bacteria in a process termed intestinal uricolysis. In states of reduced urinary excretion, there is a significant compensatory increase in intestinal UA elimination, in some gout patients the renal elimination constituting only 40 % of the total UA excretion (Hyndman et al., 2016; Sorensen & Levinson, 1975; Vaziri et al., 1995). Like in the kidney, several urate

transporters are involved in the intestinal urate handling. Some of these transporters are the same as in the kidney, with variable expression in different organs.

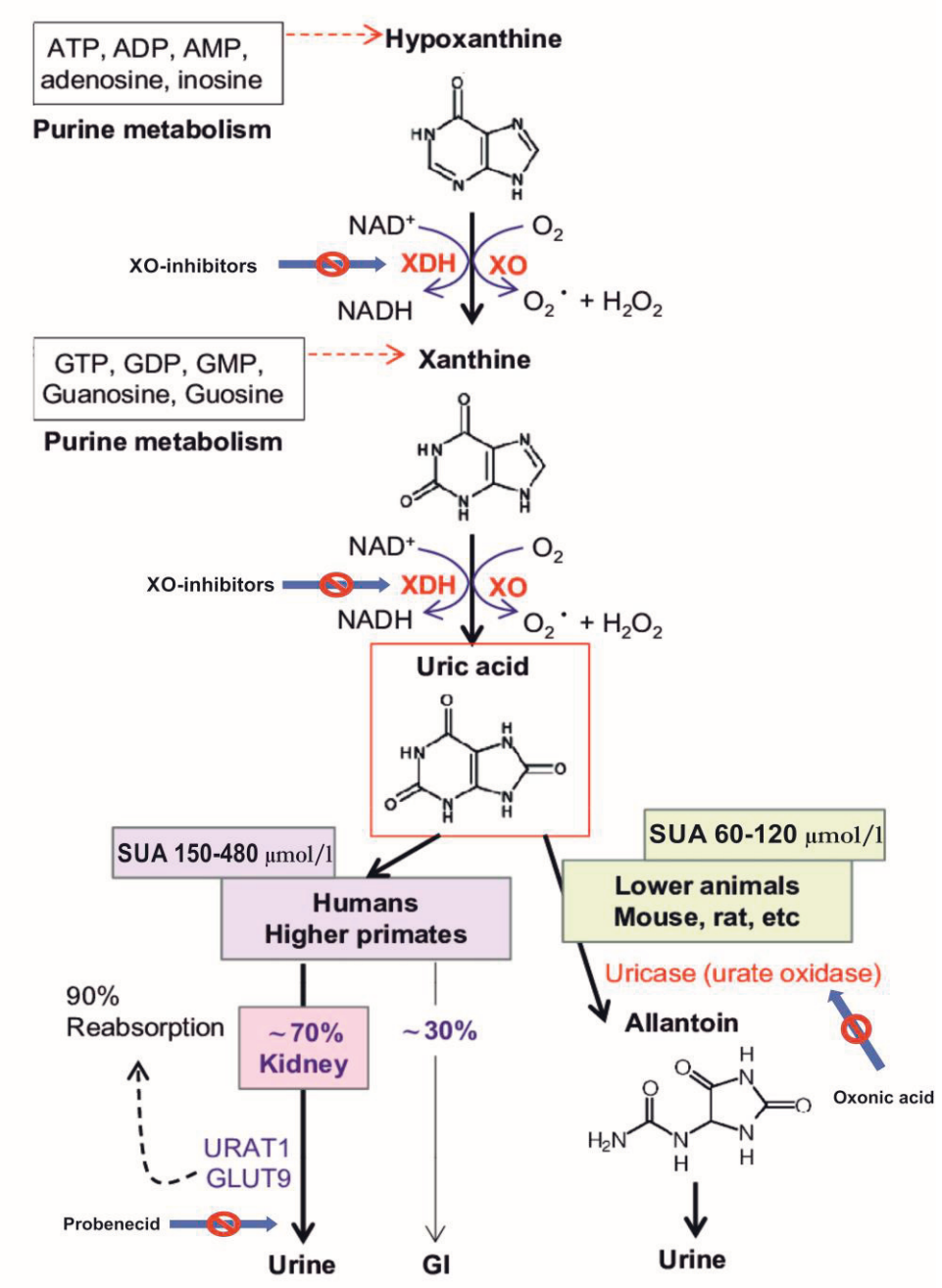

Figure 1. The generation and elimination of uric acid in humans and in other primates and the targets of uric acid lowering drugs and oxonic acid. Abbreviations explained in the text. Figure adapted from Chen et al. (2016).

1.2 Regulation of circulating uric acid levels

The circulating UA level is determined by the net balance of intake, endogenic production, and elimination of UA or its precursors. There is a significant day to day variation in individual UA levels, and a multitude of environmental, physiological, and genetic factors either enhancing the synthesis or reducing the excretion of UA can yield hyperuricemia. Renal underexcretion of UA is the most common cause for the development of hyperuricemia, and a decline in glomerular filtration rate (GFR) for any reason inversely correlates with circulating UA levels (Iseki et al., 2013; Perez-Ruiz et al., 2002).

Under normal conditions, the daily production and catabolism of UA is relatively stable, on average 300-400 mg (Maiuolo et al., 2016). Serum UA levels depend strongly on sex and age with premenopausal women having lower serum UA levels than postmenopausal women and men. In pre-pubertal children the level of UA is generally low in both sexes, while during puberty the levels increase rapidly in both sexes, but the rise is more pronounced in males (Dai et al., 2021). The effect of sex is largely attributed to the uricosuric effect of estrogen, and to a lesser extent to testosterone, having the opposite effect on circulating UA levels (Dai et al., 2021; Feig et al., 2008).

Individual serum UA variation is also compounded by genetic factors, and several single nucleotide variants that alter the function of different urate transporters have been identified (F. Butler et al., 2021). The influence of genetics is evidenced by substantial serum UA level variation across different populations and ethnicities (Bardin & Richette, 2014). For instance, New Zealand Maoris have on average higher serum UA concentrations than people of European ancestry (Roddy et al., 2007). While the carriers of the risk alleles, when also exposed to environmental and dietary risk factors, carry an increased risk for hyperuricemia, also rare monogenic conditions can cause hypo- or hyperuricemia. Renal hypouricemia is an example of an inherited condition in where pathogenic mutations in the GLUT9 or URAT1 genes influence the tubular urate transport, resulting in increased urate clearance and hypouricemia that predispose to acute kidney injury or UA nephrolithiasis (Stiburkova et al., 2013; C. Wang et al., 2019). In contrast, X-chromosomally inherited mutations in the HPRT1-gene (hypoxanthine-guanine phosphoribosyl transferase) cause Lesch-Nyhan syndrome, characterized by severe hyperuricemia, cognitive retardation, self-injurious behavior, spasticity and involuntary movements

(Nyhan, 2005). There is also a range of other dominantly and recessively inherited conditions characterized by hyperuricemia and renal disease (Shamekhi Amiri & Rostami, 2020). Urate transporters are also a target for several UA lowering medical agents, such as probenecid or angiotensin II-receptor blocker losartan, which exert their UA lowering effects by interfering with URAT1 (Bobulescu & Moe, 2012).

High-purine diet containing abundantly of red meat or seafood such as anchovies or shellfish elevate serum UA levels. Alcohol consumption accelerates hepatic break down of ATP providing the substrate for UA generation with subsequent serum UA elevation (Faller & Fox, 1982). Iron overload and hyperferritenemia have been associated with a rise in serum UA levels, while intake of vitamin B_{12} and folate associate with lower risk of hyperuricemia (Fatima et al., 2018; Zhang & Qiu, 2018). Low sodium diet, diuretic and low-dose aspirin use elevate serum UA levels by increasing renal UA reabsorption, while organic anions such as lactate, increasingly produced in ischemic conditions, elevate serum UA by competing for renal urate reabsorption (Johnson et al., 2003). Alkalization of urine promotes urinary excretion of UA (Kanbara et al., 2010).

Conditions increasing the catabolism of purine nucleic acids or cellular turnover cause excessive production of UA, but such conditions account for less than 10% of the hyperuricemic subjects. One such example is a medical condition called tumor lysis syndrome that can be induced by cancer chemotherapy or some other drugs but can also develop spontaneously if the tumor burden is large. In tumor lysis syndrome, enhanced degradation of DNA and RNA releases large amounts of UA into the circulation predisposing to acute urate nephropathy due to UA crystallization into the tubular lumen. In tumor lysis syndrome, the affected patients typically have circulating UA levels above ~900 μmol/l, and the condition should be considered as a separate entity from other conditions associated with mild to moderate subclinical serum UA elevation (Hahn et al., 2017).

1.3 Definition of hyperuricemia

The average concentration of circulating serum UA level is highly variable among individuals and even across time in each subject, making it difficult to define optimal serum UA range or a single cut-off value for hyperuricemia. Serum UA concentrations follow the Gaussian distribution and values more than two standard

deviations above the mean are statistically defined as hyperuricemic. However, statistically significant serum UA elevation is not unambiguously relevant from biological point of view, as most often biochemical serum UA levels do not correlate with disease manifestations and even lower serum UA levels within the reference range may carry a risk for the development of cardiovascular and renal complications (Bardin & Richette, 2014).

Generally, hyperuricemia is defined as serum UA concentration >420 μmol/l in men and >360 μmol/l in women (Feig et al., 2008). According to a large Finnish laboratory (Fimlab), normal plasma UA range is 155-400 μmol/l for women and 230-480 μmol/l for men.

1.3.1 Uric acid in gout and nephrolithiasis

One way to define hyperuricemia is based on the disease manifestations and symptoms, which typically occur when the concentration of UA in the blood exceeds its solubility limit and starts to form non-dissolved monosodium urate crystals. When depositing in the joints, the crystals can cause an inflammatory arthritis named gout, which is the traditional hallmark of hyperuricemia. Typically, gout presents as an acute episode of flare in the root of the first metatarsophalangeal joint, the big toe, but the disease may involve other joints as well. When prolonged, crystal accumulation induces chronic inflammation, formation of tophus and eventually irreversible joint damage. (Dalbeth et al., 2016). The gold standard method for gout diagnosis is identification of urate crystals from needle aspiration sample, and ultrasound has also been shown to be a useful diagnostic tool. Acute gout attacks are treated with nonsteroidal anti-inflammatory drugs, colchicine, or systemic/intra-articular glucocorticoids (FitzGerald et al., 2020; Richette et al., 2017).

The first historical reports from gout (originally named as podagra) date as early as 2640 B.C., a condition named as "arthritis of the rich" or "disease of kings", aptly referring to the association of gout with upper-class wealthy lifestyle (Nuki & Simkin, 2006.). Still today, the prevalence of gout follows the trends in modernization and the accompanying changes in dietary habits and cardiovascular and metabolic co-morbidities. Worldwide the prevalence of gout ranges around 1-4%, with disproportionate disease burden in men, elderly and certain ethnic groups (Dalbeth et al., 2016). The prevalence rate of gout reaches its maximum, 7%, in men over the

age of 65 (Mikuls et al., 2005). Notably, the epidemiological studies of gout are complicated by the lack of standard definition of gout and a wide variability in self-reported versus cases diagnosed by physician.

The diagnostic value of serum UA measurement in gout diagnostics is limited as 85-90% of biochemically hyperuricemic individuals do not have symptomatic gout, and on the other hand many gout patients have serum UA levels within the normal range. Furthermore, out of individuals with the highest serum UA levels (>~600 μmol/l) only about half develop gout over 15 years of follow-up (Bardin & Richette, 2014; Dalbeth et al., 2016). Thus, the individual cellular response to inflammatory urate crystals is influenced by several other factors than just circulating UA level and involves complex interactions between various cell types and inflammatory mediators (Dalbeth et al., 2016). Despite the inaccuracy in gout prediction and diagnostic, the likelihood of symptomatic gout increases in concordance with elevated serum UA concentration.

In the kidney, crystallized UA can form kidney stones, a condition named UA nephrolithiasis. The risk factors for UA nephrolithiasis are the same as in gout. In addition, hyperuricosuria, low urinary volume and low pH possess an increased risk for UA nephrolithiasis (Ngo & Assimos, 2007). Recently, it has been recognized that urate crystals can accumulate systemically in other organs as well, including aorta, coronary, vessels and the spine (Goldberg et al., 2021; Khanna et al., 2020).

1.4 Uricase mutation and the and antioxidant effects of uric acid

In most other mammals than in human and great apes, UA is only an intermediate of purine metabolism, and it is further oxidized by uricase enzyme (urate oxidase) to more soluble 5-hydroxyisourate, allantoin and ammonia which are easily excreted by the kidneys. Species possessing a functional uricase display serum UA levels in the 60-120 μmol/l range, corresponding from 1/3 to 1/7 of that in humans (Kratzer et al., 2014).

The reason for the relatively high circulating UA levels in humans is due to three nonsense mutations in the uricase gene which inactivated urate oxidase early in the Miocene epoch 8-20 million years ago (Kratzer et al., 2014). This evolutionary event may have provided potential survival benefit for the early human ancestors (Johnson,

Titte, et al., 2005). For instance, by enhancing renin-angiotensin-aldosterone system (RAAS) and increasing tubular sodium reabsorption, higher serum UA levels could have helped to maintain normal BP in ancestral times with mainly vegetarian low salt diet (Watanabe Susumu et al., 2002). Higher BP is also needed in holding the upright body posture in human and apes. Another widely referred hypothesis by Ames et al. theorized that elevated serum UA levels provided survival advantage in humans by increasing antioxidant defense (Ames et al., 1981). Indeed, UA is a potent free radical scavenger in human plasma constituting over half of the total plasma antioxidant capacity (Ames et al., 1981). Interestingly, a preceding mutation in humans that inactivated L-gulonolactone oxidase, a synthetizing enzyme for another important plasma antioxidant vitamin C, may have raised the selection pressure for an alternative antioxidant system to correct the inadequate plasma antioxidant capacity (Frei et al., 1989.) The sequential occurrence of these two events, the so called double knockout genotype, happened in a period of climate cooling and shortage of food, especially fruit supplies, with a need for more efficient mechanism for energy storing. It has been postulated that vitamin C shortage and serum UA elevation augmented human survival by increasing fructose availability and fat accumulation (Johnson et al., 2010).

The paradox over the antioxidant role of UA arises from the fact that in a specific chemical micro-environment UA can rather easily be switched from antioxidant to pro-oxidant, and vice versa (Sautin & Johnson, 2008). For instance, plasma acidification with extracellular efflux of UA can enhance the free radical scavenging properties of UA, which come more apparent in the extracellular space (Murea & Tucker, 2019). Logically, bicarbonate base inhibits the antioxidant effects of UA (Whiteman et al., 2002). Hydrophobic environment created by lipids is unfavorable for the antioxidant effects of UA as by oxidizing LDL cholesterol UA might propagate oxidative damage in atherosclerotic lesions (Sautin & Johnson, 2008). Finally, the presence of other substances such as ascorbic acid is required for the antioxidant effects of UA to manifest (Kuzkaya et al., 2005).

In the clinical context, the antioxidant potential of UA is most often addressed in association with various neurodegenerative disorders, such as Parkinson's disease, multiple sclerosis, and in acute ischemic stroke (Y.-F. Wang et al., 2018; Waring, 2002). In a large nested case-control study with over 18 000 men, subjects in the top quartile of plasma UA had 55% lower incidence of Parkinson´s disease than the men in the bottom quartile (Weisskopf et al., 2007). In inflammatory multiple

sclerosis, the beneficial effects of UA have been linked to reduced levels of peroxynitrite which, if not prevented by UA, can damage myelin and axons (Koch & De Keyser, 2006). Experimental studies have suggested that the beneficial effects of UA might be more pronounced in acute ischemic neuronal insults than in chronic conditions (Z. F. Yu et al., 1998). While this issue is still far from conclusive, the potential harm of low UA levels in these conditions has been acknowledged in the current European treatment recommendations of hyperuricemia, where long term lowering of circulating UA below 180 μmol/l is not recommended (Richette et al., 2017).

2 URIC ACID IN RENAL AND CARDIOVASCULAR DISEASE

Until late 19th century UA was mainly considered as an inert purine metabolism side product with no specific biological function nor clinical interest other than gout. In 1897, however, a British doctor Frederick Akbar Mahomed discovered a link between UA, at that time referred as "an unknown circulating toxin", and hypertension (Davis, 1897). With subsequent studies coupling UA to multiple other non-communicable chronic conditions including kidney disease, metabolic syndrome, type II diabetes, obesity, fatty liver, pre-eclampsia, and coronary artery and cerebrovascular disease, an interest over the role of UA in disease pathology re-evoked (Feig et al., 2008). Notably, in these conditions, UA levels are most often in the upper normal range or only sub-clinically elevated, currently with no indication for UA lowering interventions (Johnson et al., 2003).

The link between UA and cardiovascular and renal diseases is supported by epidemiological studies showing a similar trend pattern in the incidence and prevalence of hyperuricemia, CKD, and cardiovascular disease worldwide. For example, according to the United States National Health and Nutrition Examination Survey (NHANES), the prevalence of gout and hyperuricemia in the United States more than doubled between 1960 and 1990 (Chen-Xu et al., 2019; Zhu et al., 2011). These trends may reflect aging of the population and changed lifestyle and dietary patterns, which each bring along not only elevated UA levels, but also a cluster of other shared cardiovascular risk factors biasing the interpretation of the epidemiological observations.

2.1 Etiology and definition of chronic kidney disease

The incidence of CKD is increasing rapidly with an estimated global prevalence of around 12% (Bikbov et al., 2020; N. R. Hill et al., 2016). Furthermore, over the recent decades, CKD has constantly climbed the ranking list for the burden of disease mortality, and the burden is forecasted to still increase (Foreman et al., 2018). This

phenomenon is partly related to the ageing of the population but also to increased prevalence of cardiovascular co-morbidities, most importantly diabetes and hypertension. Irrespective of the etiology or histological presentation, hypertension is present in up to 90% of all CKD patients, the prevalence correlating with the stage of CKD: approximately 40% of patients with stage 2 CKD, and virtually all in stage 4 are hypertensive. Hypertension is considered as one of the most important risk factors for the progression of CKD, and vice versa (Muntner et al., 2010). Chronic hypertension not only aggravates CKD, but it also increases the risk for other cardiovascular manifestations, such as atherosclerosis, left-ventricular hypertrophy (LVH), heart failure and ischemic heart disease. To date, cardiovascular complications, rather than development of end-stage renal disease (ESRD) requiring renal-replacement therapy, is the major contributor to mortality in CKD patients (Foley et al., 2005; Gansevoort et al., 2013). Recognizing, preventing, and actively treating cardiovascular risk factors is therefore crucial in improving health outcomes in CKD patients.

According to the newly updated Kidney Disease: Improving Global Outcomes guidelines, CKD is defined by structural or functional abnormalities of the kidney present at least three months (Rovin et al., 2021). By this definition GFR <60 ml/min/1.73 m^2, albuminuria (>30 mg/24 hours or albumin-to-creatinine ratio >3 mg/mmol in two of three spots of urine) or the presence of histological, radiological, or biochemical markers of kidney damage is diagnostic for CKD. Glomerular filtration rate should be estimated (eGFR) by using serum creatinine concentration and transform it by using CKD-Epidemiology Collaboration (CKD-EPI) equation, which also corrects for age race and sex (Levey et al., 2009). Of note, a recent revision of the CKD-EPI formula for eGFR only needs the correction for sex (Delgado et al., 2021). In general and in high-risk populations lower eGFR and greater albuminuria are associated with higher all-cause and cardiovascular mortality, highlighting the relative importance of these easily accessible non-costly markers in the clinical evaluation of CKD patients (Matsushita et al., 2010; van der Velde et al., 2011).

Chronic renal disease can be classified into five stages according to the level of GFR (Table 1). The mildest stages one and two require evidence of kidney damage, practically hematuria, proteinuria, or albuminuria, while from stage three the diagnosis can be set by GFR only.

Table 1. Stages of chronic kidney disease (KDIGO-guidelines 2021).

Stage	Description	GFR (mL/min/1.73 m^2)
1	Normal or high	≥90
2	Mildly decreased	60-89
3a	Mildly to moderately decreased	45-59
3b	Moderately to severely decreased	30-44
4	Severely decreased	15-29
5	Kidney failure	< 15 (or dialysis)

2.1.1 Renal pathology in chronic kidney disease

Irrespective of the etiology, the final endpoint in CKD is the destruction of normal renal histological architecture due to the development of glomerulosclerosis, vascular sclerosis and interstitial fibrosis (Fogo, 2007; H. T. Yu, 2003). Notably, interstitial fibrosis correlates better with the degree of functional renal impairment and disease prognosis even when the primary insult is of glomerular origin (Schainuck et al., 1970).

The renal fibrogenic process is basically an impolitic response to kidney injury which triggers a cascade of reactions aiming to recover the initial damaging event. An imbalance between cell proliferation and apoptosis leads to a loss of normal resident glomerular and interstitial renal cell population (Savill, 1999). Apoptosis is stimulated by tissue hypoxia resulting from hypoperfusion of the microvasculature (Khan et al., 1999; H. T. Yu, 2003). The apoptotic loss of podocyte cells, which have a key role in maintaining normal glomerular permselectivity, contributes to the progression of glomerular injury, partly due to a secondary insult to the adjacent endothelial and mesangial cells (Ichikawa et al., 2005). An infiltration of

inflammatory lymphocytes, macrophages and mast cells promote the production of ROS, profibrotic cytokines (most importantly transforming growth factor-β, TGF-β) and growth factors, implicating the crucial role of oxidative stress and various vasoactive substances in CKD (H. T. Yu, 2003). The accumulation of cytokines induces generation of extracellular matrix proteins mainly by the interstitial (myo)fibroblasts but also by the glomerular mesangial cells. Activated myofibroblasts are the major source of collagen and smooth muscle cell alpha-actin (SMA), the latter used as an indicator of myofibroblast activity and predictor of renal dysfunction both in human and experimental renal disease (Fogo, 2007). Renal fibrosis is further accelerated by reduced levels of various antifibrotic factors (Eddy, 2005).

After the initial insult, the fibrotic pathway is driven by hemodynamic alterations in the kidney. Experimental studies with subtotally nephrectomized rats have shown that the loss of functioning nephrons leads to adaptive hyperfiltration of the remnant glomeruli resulting in glomerular hypertension and progressive GFR decline (Hostetter et al., 1981). Stretching of the glomerular capillary tuft and the adjacent mesangial cells induces mesangial cell proliferation with the overexpression of cytokines that enhance glomerulosclerosis (Suda et al., 2001). There is evidence that similar compensatory hyperfiltration propagates GFR decline in human diabetic nephropathy as well (Imanishi et al., 1999), and that life-style and pharmacological interventions that decrease hyperfiltration can effectively ameliorate the development of glomerulosclerosis (Fogo, 2007; Nath et al., 1986). Activation of RAAS contributes to CKD progression directly by inducing systemic and intrarenal hypertension and via profibrotic and proinflammatory actions of angiotensin II and aldosterone.

2.1.1.1 Vascular changes in chronic kidney disease

Atherosclerosis is a frequent finding in CKD already at the very early stages of the disease (Schiffrin et al., 2007). As in the general population, the development of atherosclerotic lesions is driven by traditional risk factors including systemic hypertension and overactivity of the sympathetic nervous system, while anemia, hyperhomocystinemia and abnormal calcium/phosphate metabolism are characteristic features in CKD (Converse et al., 2010). In addition to these risk factors, non-traditional risk factors such as low-level inflammation, increased levels of oxidized lipids, and oxidative stress further accelerate disease progression.

Endothelium, a thin single cell layer in the inner arterial wall separating the blood stream from the underlying smooth muscle, is a dynamic organ that regulates vascular tone by synthesizing and releasing various vasoactive substances in response to hemodynamic stimuli (Figure 2). Compromised endothelial function, referred to as endothelial dysfunction, critically participates in the mechanisms that lead to the development of atherosclerotic lesions and progression of CKD (Cahill & Redmond, 2016). Impaired regulation of the arterial tone is largely attributed to impaired NO-mediated endothelium-dependent vasorelaxation. Nitric oxide is synthetized by the endothelial cells from its precursor L-arginine by the constitutive endothelial NO synthase (eNOS). By activating guanylate cyclase, NO increases the generation of cyclic guanosine monophosphate (cGMP) which reduces intracellular free calcium (Ca^{2+}) concentration and relaxes the smooth muscle. In addition to vasorelaxation, the anti-thrombotic and anti-inflammatory properties of NO can oppose atherosclerotic process. The release of endothelial NO can occur in response to a wide range of different physical and chemical stimuli and various molecules, of them acetylcholine (Ach) being the most frequently used assay for studying NO-mediated endothelial responses (Vanhoutte et al., 2017). The generation of NO, and arterial vasorelaxation, are often compromised under hypoxic conditions, largely due to excess generation of ROS that interfere with NO-mediated processes.

Other vasodilatory agents released by the endothelium include prostacyclin (PGI_2), which mediates its actions via cyclic adenosine monophosphate (cAMP) and activation of the prostaglandin I_2 receptor (Félétou et al., 2010). A third pathway mediating endothelium-dependent vasorelaxation is attributed to endothelium-derived hyperpolarizing factor (EDHF), which causes vasorelaxation without detectable changes in intracellular cyclic nucleotide (cGMP and cAMP) contents and is therefore not blocked by inhibiting NO and prostacyclin pathways (Vanhoutte et al., 2017). The identity of EDHF is still not fully characterized, but it is often considered as endothelial cell potassium (K^+) channel opener. In brief, the efflux of K^+ from endothelial cells mainly through intermediate- and small-conductance Ca^{2+}-activated K^+ channels (K_{Ca}), causes hyperpolarization of the endothelial cells which is further transmitted to the adjacent vascular smooth muscle cell (VSMC) via myoendothelial gap junctions. Elevated K^+ concentration in the intercellular space leads to activation of K^+ channels and Na^+/K^+-ATPase in the VSMC inducing hyperpolarization, inhibition of Ca^{2+} inflow, and vascular relaxation. Various substances, including products of cytochrome P450 metabolism such as epoxyeicosatrienoic acids (EET) have been proposed as EDHF (Félétou &

Vanhoutte, 2006). A third type of K_{Ca}, named large-conductance K_{Ca} (BK_{Ca}) is mainly expressed in the VSMC, and it mediates vasodilatation endothelium-independently. BK_{Ca} cause hyperpolarization of the VSMC by inducing K^+ efflux in response to intracellular calcium sparks (Félétou, 2009). The opening state of the BK_{Ca} can be modulated by various physiological or pharmacological agents. For instance, 20-hydroxyeicosatetraenoic acid can inhibit BK_{Ca}, while H_2O_2 and ROS can either activate or inactivate the channels (Félétou, 2009; Gutterman et al., 2005; Yin et al., 2017).

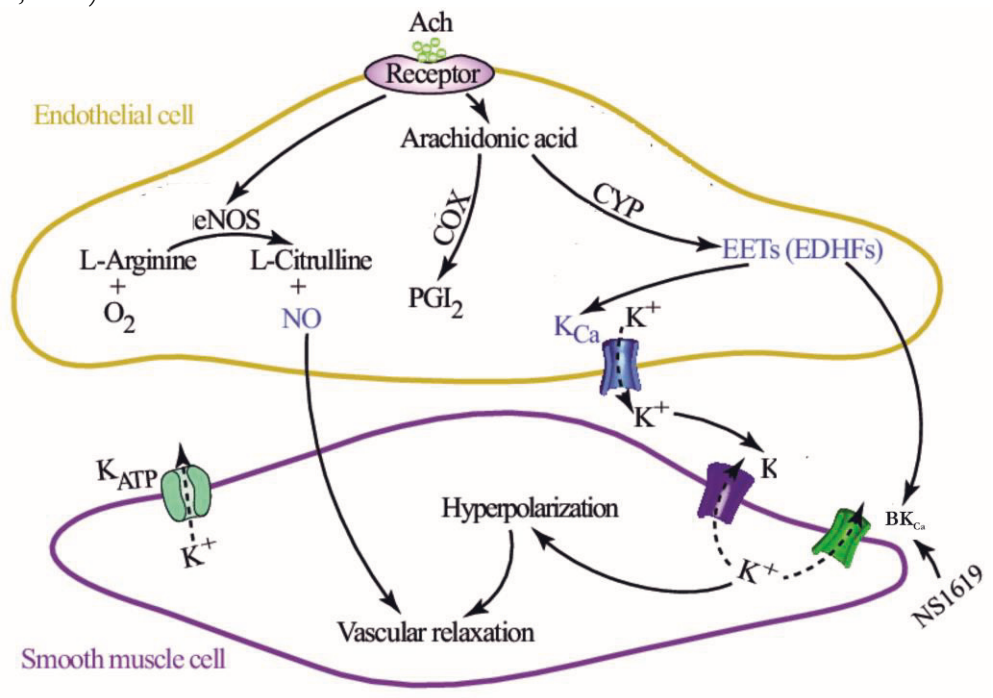

Figure 2. Principal mechanisms of endothelium-mediated relaxation of vascular smooth muscle cell and the targets of agents used in the vasorelaxation studies. Figure adapted from Yin et al (2017).

The relative contribution of different mediators of vasorelaxation varies in different vascular beds. In general, in conduit-size arteries the vasorelaxation is mostly mediated via NO, while the role of EDHF increases along with the decrease in vessel diameter (Félétou et al., 2010).

Chronic renal disease is also characterized by remodeling of the conduit size and small resistance arteries, contributing to hemodynamic changes observed in CKD. (P. Kööbi et al., 2003). Vascular remodeling is featured by increased wall stiffness

due to medial calcification, increased extracellular matrix content and VSMC hyperplasia (London et al., 2005).

2.1.1.2 Cardiac load

Left ventricle hypertrophy is a frequent finding in CKD patients and increased left ventricle mass is one of the most important predictors of adverse cardiovascular outcome in CKD and even in the general population (Amann et al., 1998a; McCullough et al., 2016). In patients starting renal replacement therapy the prevalence of LVH has been reported to be up to 80%, and there is a progressive increase in left ventricular mass in conjunction with the hemodialysis therapy (Amann et al., 1998a; Hüting et al., 1988). Causes for LVH in CKD are multifactorial including anemia, distorted mineral metabolism, hypervolemia, and hypertension. Persistent hypervolemia predisposes to LVH already in the early stages of the disease when GFR is still retained (Amann et al., 1998b). In addition, hemodynamic alterations including increased peripheral arterial resistance and reduced compliance of the large arteries further add cardiac load.

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP), produced predominantly by cardiac atria and ventricles, regulate sodium and volume homeostasis, BP, and vascular function (Volpe, 2014). While ANP represents the physiological natriuretic peptide, BNP comes to prominence in pathophysiological stress situations. Nevertheless, the secretion of both hormones is stimulated by increased cardiac wall stress in response to excessive fluid volume and can therefore be regarded as an index of volume load. In CKD, circulating levels of natriuretic peptides are frequently increased even in the absence of cardiac dysfunction, a phenomenon reflecting an adaptive response to sodium retention and hypervolemia (Volpe, 2014). In the kidney, natriuretic peptides increase urinary excretion of sodium and water but also of other retained solutes, such as urea, potassium, and phosphate. They also optimize renal function by increasing renal blood flow and filtration. In addition, ANP may protect against angiotensin II- or noradrenaline-induced myocardial remodeling in the rat (Calderone et al., 1998; Fujita et al., 2013). In the vasculature, ANP can reduce intravascular volume by increasing endothelial permeability, while BNP can improve endothelial cell regeneration after ischemic insult (Kuhn, 2012). Measuring plasma BNP levels can be used as a tool to assess cardiovascular risk in subjects with CKD (Sakuma et al., 2010; Yasuda et al., 2012). Increased pressure and volume overload, as indicated by increased expression of

heart ventricle ANP and BNP mRNAs already before the BP elevation, has been observed in the 5/6 NX model of experimental CKD (P. Kööbi et al., 2003).

2.2 Hyperuricemia and chronic kidney disease

Addressing the role of UA in renal pathology is challenging, as the hallmarks of renal disease, enhanced XO activity and decline in GFR, elevate circulating UA. Indeed, the prevalence of hyperuricemia exceeds 60% in stage 3-4 CKD, and CKD is one of the most common risk factors for gout (Juraschek et al., 2013; Madero et al., 2009). Furthermore, cardiovascular co-morbidities, including cardiac hypertrophy and dysfunction are prevalent in hyperuricemic CKD patients, both contributing to and resulting from CKD. Several studies have found an association between hyperuricemia and LVH both in the general and in the CKD populations (Chiu et al., 2020; Cuspidi et al., 2017; Gromadziński et al., 2015; Kim et al., 2021). A combination of higher UA levels and left ventricle mass may also predict the progression of decline in renal function in patients with moderate to severe CKD (S.-C. Chen et al., 2013).

In subjects with intact renal function, high circulating UA level is generally considered as an independent risk factor for both incident and end stage renal disease (Hsu et al., 2009; L. Li et al., 2014; Weiner et al., 2008). Furthermore, elevated childhood UA levels have predicted the development of hypertension later in life (Alper et al., 2005). The evidence regarding the role of UA in the progression of pre-existing kidney disease is somewhat more conflicting: while some studies have concluded that UA is an independent risk factor for disease progression, others have not supported this view (Srivastava et al., 2018; Sturm et al., 2008). In subjects with immunoglobulin-A nephropathy, higher circulating UA levels have been correlated with histological renal parameters, most importantly tubulointerstitial damage (Myllymäki et al., 2005). In the most severely affected patients with ESRD, both high and low UA levels have conveyed an increased cardiovascular risk (Suliman et al., 2006). In two large meta-analyses, one including CKD patients and the other a heterogenous population, a positive dose-dependent association between UA and cardiovascular mortality were detected (Luo et al., 2019; Rahimi-Sakak et al., 2019). Most comprehensive umbrella review including a few hundred systematic reviews, meta-analyses and Mendelian randomization studies covering 136 different health

outcomes, concluded that convincing evidence over the role of UA in disease states only exists for gout and nephrolithiasis (X. Li et al., 2017).

2.2.1 Evidence from the experimental models of hyperuricemia

Autopsy samples from humans with gout almost exclusively feature variable degrees of histological renal scarring consisting of glomerulosclerosis, interstitial fibrosis, and arteriolosclerosis with accumulation of urate crystals focally in the outer medullary region, a condition termed analogously to gouty arthritis, as "gouty nephropathy" (Talbott & Terplan, 1960). Furthermore, before the availability of UA lowering treatments up to one-fourth of gouty patients developed ESRD (Johnson et al., 2013). In these preliminary observations, the association of UA and gout with renal pathology was largely attributed to the presence of focal renal urate crystals, but they did not explain the diffuse renal scarring observed in gouty subjects, nor the finding that urate crystals were found also in subjects without kidney disease. This controversy gave birth to an alternative hypothesis suggesting a non-mechanic pathway for UA associated renal disease and emphasized the need for an experimental model of hyperuricemia (Johnson et al., 2013).

To study the causal role of UA in cardiovascular and renal disease, an experimental model of non-gouty hyperuricemia was developed in 2001 and since then it has been repeatedly employed in rats with normal and impaired renal function (Kang, 2002; Khosla et al., 2005; Mazzali et al., 2002; Mazzali, Hughes, et al., 2001; Mazzali, Kim, et al., 2001; Nakagawa et al., 2003; Sanchez-Lozada et al., 2005; Sánchez-Lozada et al., 2002). In this model, rats fed with dietary 2% Oxo supplement for 4-7 weeks develop mild hyperuricemia, and they also present with hypertension and histological non-gouty renal scarring. These studies have suggested an independent role of UA in the development of the diseases as lowering circulating UA with XO-inhibitors allopurinol or febuxostat or by administering uricosuric agent benziodarone has prevented the changes (Mazzali et al., 2002; Mazzali, Hughes, et al., 2001; Sanchez-Lozada et al., 2005; Sánchez-Lozada, Soto, et al., 2008).

Previous experimental studies have suggested that the trigger for UA induced hypertension and renal disease is the activation of systemic and renal RAAS and reduced abundance of NO. This has been indicated by increased plasma renin and aldosterone, increased number of renin-positive cells in the kidney, decreased NOS

synthesis in the juxtaglomerular apparatus, and reduced serum and urinary NO metabolite concentration in hyperuricemic rats with retained or impaired renal function (Eräranta et al., 2008, 2008; Johnson, Rodriguez-Iturbe, et al., 2005; Khosla et al., 2005; Mazzali, Hughes, et al., 2001; Mazzali, Kim, et al., 2001; Sanchez-Lozada et al., 2005). The role of RAAS and NO-pathways in hyperuricemia-associated renal disease has been further supported by the observations that blocking RAAS by administering angiotensin receptor blocker losartan or enhancing the NOS system with L-arginine have normalized the BP and other changes associated with the hyperuricemic state (Mazzali et al., 2002; Mazzali, Hughes, et al., 2001; Nakagawa et al., 2003).

The role of increased oxidative stress has been implicated in the pathophysiological changes associated with experimental hyperuricemia. According to this theory, increased oxidant burden might reduce the availability of NO, which would induce renal vasoconstriction, hypoperfusion, ischemia and inflammation, ultimately causing renal fibrosis (Mazzali et al., 2002; Sanchez-Lozada et al., 2005). Furthermore, structural changes in the renal vasculature, namely afferent arteriolopathy, might contribute to local and systemic BP elevation in hyperuricemia (Sánchez-Lozada, Soto, et al., 2008). In cell cultures, UA was found to induce rat aortic VSMC proliferation and COX-2 expression, while a one week study found that UA reduced NO production in cultured bovine aortic endothelial cells (Kang, 2002; Khosla et al., 2005; Rao et al., 1991). The association of high circulating UA levels with dysfunctional glomerular perfusion has also been implicated in humans (Uedono et al., 2015). Finally, UA was also found to trigger epithelial-to-mesenchymal transition in cultured rat tubular cells, suggesting a novel pathway for the actions of UA in the kidney (Ryu et al., 2013).

2.3 Arterial stiffness and cardiovascular risk in human

Arterial BP is the product of cardiac output (CO) systemic vascular resistance (SVR). Furthermore, CO equals the volume of blood that the left ventricle ejects to the aorta during systole (stroke volume, SV) multiplied by heart rate (HR) during each minute. Vascular resistance is influenced by vessel length, distensibility and viscosity of blood, but its main determinant is the radius of the vessel. Most of the peripheral arterial resistance is attributed to resistance arteries (diameter less than 400 μm),

while larger arteries act more like a conduit system. The smallest capillary vessels do not play a role in the regulation of the vascular resistance.

Arterial pressure between the systolic and diastolic phases of the heart cycle varies in a pulsatile manner. Within each heartbeat, arterial pulse and the backward reflected wave from the periphery generate the arterial pulse wave, as described in Figure 3. Pulse pressure is the difference between the systolic and diastolic BP, and ΔPP (augmentation pressure) is the difference between the first inflection point (when the reflected wave starts to influence central pressure) and the peak of the systolic pressure wave. The form of the pulse wave reflects the compliance of the arteries: in compliant arteries the reflected wave returns to the aortic root during the diastole, while stiffening of the large arteries leads to earlier return of the reflected wave. In the former case the velocity of the reflected wave, indicated as PVW is low, and in the latter PWV is higher.

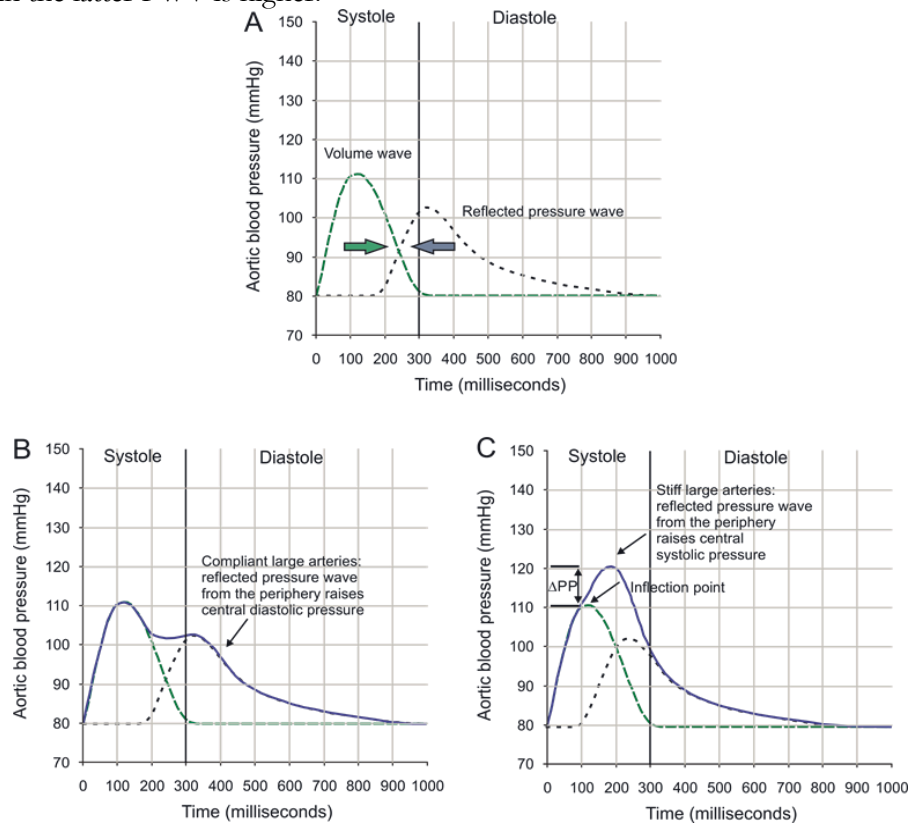

Figure 3. Arterial pulse and the backward reflected wave (A), the aortic pulse wave form in an individual with compliant arteries (B), and in an individual with stiff arteries (C). ΔPP is the increase in pulse pressure due to the reflected wave, i. e. augmentation pressure.

Pulse wave velocity increases in concordance with narrowing of the arterial lumen and compliance, and its measurement is considered as the gold standard in the evaluation of arterial stiffness (Laurent et al., 2006). Notably, PWV can be measured in various sites of the arterial tree. While carotid-femoral PWV is the measure of aortic stiffness, carotid-radial PWV, brachial-ankle PWV and brachial-radial PWV measure both central and peripheral arterial stiffness. Decreased large arterial compliance is generally considered as an independent predictor of all-cause and cardiovascular mortality (Laurent et al., 2003; Mattace-Raso et al., 2006; Vlachopoulos et al., 2010).

Each of the determinants of BP are affected by many local, neural, renal, and humoral factors. While short-term control of BP is predominantly regulated by direct effect of sympathetic and parasympathetic nerve-endings on the arterial wall or indirect renal renin secretion, the kidneys play a key role in long-term BP maintenance. In addition, vascular endothelium regulates the peripheral vascular resistance and BP by responding to shear stress on the vessel wall. The endothelium itself does not have a capability to alter the contractile state of the vessel, but it influences the contractile state of the underlying smooth muscle by releasing various vasoactive transmitters (Cowley, 1992; Sandoo et al., 2010).

2.3.1 Uric acid and arterial stiffness

The association between UA and arterial stiffness has been evaluated in numerous cross-sectional and prospective studies, with conflicting results (Albu et al., 2020). In prospective studies with four to six years of follow-up, some reports found a direct relationship between UA and arterial stiffness, but others did not (Canepa et al., 2017; Maloberti et al., 2019; Nagano et al., 2017). In subjects from the Framingham heart study with low cardiovascular risk, UA was linked to arterial stiffness, as evaluated by carotid-femoral PWV. The association remained, though weaker, when the medicated hypertensive patients were excluded from the analysis (Mehta et al., 2015). In hypertensive patients, higher UA levels have been associated with higher carotid-femoral PWV and brachial-ankle PWV (An et al., 2020; Liu et al., 2019). Furthermore, in untreated hypertensive subjects plasma UA correlated with carotid-femoral PWV, but not with 24-h systolic or diastolic BP, and after correction for albuminuria and other covariates the association was no longer significant (Mulè et al., 2014). Some of the studies have observed the association between UA and PWV only in women, and some only in men. It has also been

suggested that there is a threshold for serum UA association with arterial stiffness (Canepa et al., 2017).

2.4 Uric acid and the metabolic syndrome

In recent decades, there has been a growing interest for the extra-renal manifestations and associations of hyperuricemia, most importantly metabolic syndrome, and its individual components; obesity, dyslipidemia, hypertension, and insulin resistance. Historically, in subjects with the metabolic syndrome, hyperuricemia has been considered secondary to hyperinsulinemia as insulin decreases renal UA excretion (Quiñones Galvan et al., 1995). On the other hand, glucosuria decreases tubular UA absorption with an opposite effect on circulating UA levels (Johnson et al., 2009). It has been suggested that UA might also directly influence glucose metabolism and by this means play a role in the pathogenesis of the metabolic syndrome and other associated conditions, such as fatty liver disease and obesity (Johnson et al., 2009).

In the adult population, the risk of developing type 2 diabetes has been reported to increase dose-dependently by 6 to 20% for each 60 μmol/l increment in circulating UA concentration (Kodama et al., 2009; Lv et al., 2013; Sluijs et al., 2015). A recent meta-analysis acknowledged the association between UA and pediatric metabolic syndrome (Goli et al., 2020). Evidence showing that hyperuricemia often precedes the development of type 2 diabetes supports a causal relationship between these conditions (Bhole et al., 2010), but this view has not been confirmed by other studies (Sluijs et al., 2015).

According to one relatively new hypothesis, the effects of UA might, independently from energy intake or weight gain, specifically relate to fructose. Fructose is a sugar derived from sucrose (table sugar) or high fructose corn syrup, both consisting of glucose and fructose (about 50% of each). Fructose is a major constituent in processed food and soft drinks that are abundantly consumed in the western diet (J. W. J. Choi et al., 2008; Johnson et al., 2009). Unlike other sugars, fructose has a unique capability to facilitate hepatic UA generation and elevate serum UA as evidenced by elevation is serum UA levels after ingestion of fructose (Perheentupa & Raivio, 1967). The metabolism of fructose increases ATP turnover,

AMP accumulation, and purine degradation and it can also directly stimulate UA production from amino acids.

Like in CKD, the potential actions of UA in the metabolic syndrome might be coupled to reduced availability of NO and increased oxidative stress. In cultured human endothelial cells, UA was found to inhibit insulin-induced eNOS activity and subsequent NO production (Y. Choi et al., 2014). Uric acid might also directly induce oxidative stress in pancreatic β-cells (Zhang et al., 2013). High fructose-induced hyperuricemia has been found to induce renal effects similar to those seen in experimental CKD, and the effect that was normalized by lowering UA with febuxostat (Sánchez-Lozada et al., 2007; Sánchez-Lozada, Tapia, et al., 2008). There is also evidence that lowering UA with allopurinol normalizes endothelial dysfunction in type 2 diabetes (R. Butler et al., 2000).

2.5 Long-term treatment of hyperuricemia

Non-pharmacological lifestyle modification can be recommended with low threshold to all subjects with symptomatic gout, and to non-symptomatic subjects with cardiovascular risk factors, with or without biochemically confirmed hyperuricemia. Lifestyle interventions include maintenance of normal weight, physical activity, smoking cessation, and avoidance of animal-derived purine-rich food, high fructose containing beverages, salt, alcohol, and medications that raise UA levels. For a large fraction of hyperuricemic individuals, however, these interventions are insufficient resulting in a maximum 20% decrease in the circulating UA concentration (FitzGerald et al., 2020).

Currently there is enough evidence for the long-term treatment of hyperuricemia only in patients with symptomatic gout, while the treatment of asymptomatic hyperuricemia is recommended only in specific circumstances. These conditions include patients with very high circulating UA levels (cut-off serum UA level varying from ~470 μmol/l to ~770 μmol/l), high renal UA excretion (>1100 mg/day), or patients receiving chemo- or radiotherapy with risk for tumor lysis syndrome (Brucato et al., 2020). Aside from these relatively small patient entities, the question whether patients with asymptomatic hyperuricemia should be treated and which UA threshold levels should be applied, is still unresolved. Due to multiple co-morbidities, hyperuricemic subjects often possess an increased risk for polypharmacy, which

brings along not only the risk for severe side effects but also non-adherence to urate lowering therapy.

Several large clinical randomized controlled studies and meta-analyses have been carried out to solve the issue whether patients with CKD would benefit from urate lowering drugs (Badve et al., 2020; Brucato et al., 2020; Doria et al., 2020; Kimura et al., 2018; Sato et al., 2019; Tanaka et al., 2020; Tsukamoto et al., 2021). Two rather newly published randomized, double-blind, placebo-controlled studies explored the efficiency of two to three year allopurinol treatment in slowing down the progression of the decline in renal function in normouricemic non-gouty CKD patients (Badve et al., 2020; Doria et al., 2020). The first study consisted of stage 3 and 4 CKD patients and the second of stage 2-3 CKD patients with type 1 diabetes. Neither of these studies found benefit from allopurinol treatment in comparison with placebo, concluding that UA is not causally linked to CKD progression. Similar conclusions were drawn from a trial using febuxostat in CKD stage 3 patients with asymptomatic hyperuricemia for two years, in whom febuxostat did not mitigate the decline in renal function as indicated by eGFR (Kimura et al., 2018). However, in a review by Sato et al. summarizing the results from 22 randomized controlled trials assessing the effect of UA lowering treatment in hyperuricemic patients with CKD, the conclusion was that especially from stage 3 CKD onwards, treatment of hyperuricemia may slow down the progression of renal disease (Sato et al., 2019). In older hyperuricemic patients with pre-existing or high risk for cardiovascular disease, treatment of hyperuricemia with febuxostat was found to reduce cerebral, cardiovascular and renal events (Kojima et al., 2019). The beneficial effects of XO inhibitors in subjects with cardiovascular disease have been coupled to reduced oxidative stress and improved endothelial function (Higgins et al., 2012).

According to the existing guidelines from the American College of Rheumatology (ACR, 2020) and the European League Against Rheumatism (EULAR, 2016), testing for or treatment of hyperuricemia is not indicated in the absence of gout or nephrolithiasis, but cardiovascular risk factors should be actively screened and managed (FitzGerald et al., 2020; Richette et al., 2017). According to the EULAR recommendation, the first-line and the most effective pharmacological treatment of chronic hyperuricemia is with XO-inhibitors allopurinol, oxypurinol or, in case of inefficiency or intolerance to the former, non-purine selective XO-inhibitor, febuxostat (Richette et al., 2017). Allopurinol should be cautiously used and with dose reduction especially in patients with decreased renal function, as in around 1%

of patients, it can induce a potentially lethal, serious cutaneous adverse reaction including rash with eosinophilia, Steven-Johnson syndrome, and toxic epidermal necrosis. (Richette et al., 2017.) In subjects with a high risk for cardiovascular disease allopurinol is preferred from febuxostat as in this population the use of febuxostat has been reported to increase all-cause and cardiovascular mortality (White et al., 2018). If a proper control of UA levels is not reached with XO inhibitors, the uricosuric agents benzbromarone or probenecid can be used either alone or in combination with allopurinol (Richette et al., 2017). Uricosurics should be avoided in patients with renal caliculi or advanced CKD. In cases with unsuccessful conventional therapy, peglotigace, a genetically engineered Escheria coli-derived mammalian uricase may be an effective drug of choice (Sundy et al., 2008). Due to parenteral administration and a risk for infusion reactions, the use of uricases is limited to severe and recurrent gout.

In medically treated patients, the target serum UA level is generally considered at <360 μmol/l in patients with symptomatic hyperuricemia, or <300 μmol/l in subjects with severe gout (frequent attacks, chronic arthropathy) (FitzGerald et al., 2020; Richette et al., 2017). Some, but not all, experts recommend early medical intervention in CKD patients with an aim to delay or even improve deterioration of renal function (Kielstein et al., 2020). Notably, hypouricemia <180 μmol/l should be avoided due to the possible negative health effects (Richette et al., 2017).

3 AIMS OF THE STUDY

The purpose of the present series of experimental studies (studies I, II, III) was to examine the cardiovascular and renal effects of 9 weeks of 2% oxonic acid diet-induced hyperuricemia in normal control and 5/6 NX rats, with a special interest on systemic and renal oxidative stress markers (aims 1-3, same rats employed). The clinical study (study IV) investigated the association of plasma UA levels with several hemodynamic variables in normotensive and never-medicated hypertensive volunteers by applying the non-invasive methods whole-body impedance cardiography and continuous radial tonometric pulse wave analysis (aim 4).

The specific aims of the study were:

1. To examine the effect of hyperuricemia on the control of carotid artery tone, systemic oxidative stress, and plasma antioxidant capacity.

2. To investigate the influence of elevated UA levels on mesenteric artery tone and structure and cardiac load.

3. To evaluate the effects of hyperuricemia on indices of kidney damage, inflammation, and oxidative stress.

4. To study the association of plasma UA levels with several non-invasively measured hemodynamic variables, most importantly PWV, in normotensive and hypertensive volunteers with UA levels predominantly within the normal range.

4 MATERIALS AD METHODS

4.1 Study design in the experimental studies (studies I, II, III)

Male Sprague-Dawley rats were housed under standard animal laboratory conditions with free access to water and food pellets (Lactamin R34, AnalyCen, Lindköping, Sweden) containing 0.9% calcium, 0.8% phosphorus, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 12550 kJ/kg energy, 16.5% protein, 4.0% fat, 58% nitrogen-free extract, 3.5% fiber, 6.0% ash and 10% water. At the age of 8 weeks, the rats were anesthetized with ketamine/diazepam (75 and 2.5 mg/kg, intraperitoneally, respectively) and the NX operations (study week 0) were carried out by the removal of upper and lower poles of the left kidney and the whole right kidney. The kidneys of the Sham rats were decapsulated. Postoperative antibiotics (metronidazole 60 mg/kg and cefuroxime 225 mg/kg) and analgesic (buprenorphine 0.2 mg/kg) were given.

Three weeks after the operations, at the age of 11 weeks (study week 3), the rats were assigned to 4 groups (n=10-12 in each group): Sham, Sham-Oxo, NX and NX-Oxo with comparable systolic BPs, body weights and urine volumes in both Sham and both NX groups (Figure 4). After division, the chow for Sham-Oxo and NX-Oxo groups was supplemented with 2.0% Oxo (20 g/kg chow, Sigma-Aldrich Chemical Co, St. Louis, MO, USA), whereas the Sham and NX groups continued the normal diet for 9 weeks. Hyperuricemia was confirmed by tail vein sampling at study week 5, and 24-hour urine output and fluid consumption were evaluated in metabolic cages prior to and at the end of the Oxo feeding. Systolic BP was measured at 28°C by the tail-cuff method as the averages of five recordings in each rat (Model 129 Blood Pressure Meter; IITC Inc., Woodland Hills, CA, USA).

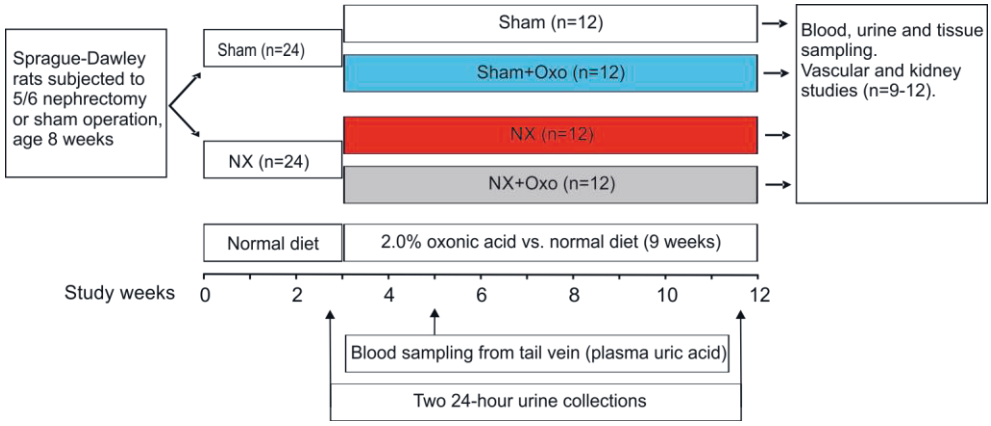

Figure 4. Flowchart of the studies I, II and III. NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat.

After 9 weeks of Oxo diet (study week 12), the rats were anesthetized with urethane (1.3 g/kg) and blood samples from cannulated carotid artery were drawn into appropriate tubes with either heparin or EDTA as anticoagulants. The hearts and the kidneys were harvested and weighed. The heart ventricles were snap-frozen in liquid nitrogen-cooled isopentane and stored at -70ºC until the extraction of the total RNA. The kidneys were frozen in isopentane at -40ºC and stored at -80ºC or fixed in 4% formaldehyde for 24 hours and embedded in paraffin, as appropriate. The study was approved by the Animal Experimentation Committee of the University of Tampere, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland, and conforms to the Guiding Principles for Research Involving Animals.

4.1.1 Biochemical blood and plasma determinations

Plasma creatinine and urea were measured using standard clinical chemical methods (Cobas Integra 800 Clinical Chemical Analyzer, Roche Diagnostics, Basel, Switzerland) and plasma UA was measured using an enzymatic colorimetric method (Prætorius & Poulsen, 1953). Plasma renin activity was assessed using a GammaCoat assay (Diasorin SpA Saluggia, Italy), and the other determinations were carried out as described earlier (Jolma et al., 2003; P. Kööbi et al., 2003). Plasma triglycerides, total and high-density lipoprotein (HDL) cholesterol concentrations were analyzed using Cobas Integra 800 automatic analyzer (Hoffman-La Roche, Basel, Switzerland)

and non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

Plasma TRAP was measured using luminol-enhanced chemiluminescence method (study I). The method is based on peroxyl radical production by decomposition of 2,2-azo-bis(2-aminopropane) hydrochloride (ABAP; Polysciences, Warrington, PA, USA), as previously described (Alanko et al., 1999; Dugué et al., 2005). In the measurement, the tested compounds from 2 to 20 nmol per assay were subjected to peroxyl radicals produced at a known and constant rate by the thermal decomposition of ABAP at 37°C. The presence of free radicals in the reaction mixture was monitored by luminol-enhanced chemiluminescence. The composition of the reaction mixture in cuvette was: 475 ml of 100 mM phosphate buffer, pH 7.4 in saline; 50 ml of 400 mM ABAP and 50 ml of 10 mM luminol in 100 mM borate buffer, pH 10. The solvents used had themselves no radical-scavenging properties. The addition of an antioxidant dissolved in 20 ml of 100 mM phosphate buffer in saline, pH 7.4 to the reaction mixture extinguished the chemiluminescence. The duration of extinction had a linear correlation to the radical trapping capability of the tested compound. Water soluble tocopherol, Trolox, which is known to trap two radicals per molecule (stoichiometric factor 2.0), was used as a standard. For each phenolic compound the plots of the extinction of chemiluminescence versus the concentration were drawn and stoichiometric factors were calculated.

4.1.2 Kidney heme-oxygenase-1 and collagen I mRNA expression with real-time quantitative PCR (study III)

Total RNA was isolated from rat kidney tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription of RNA was performed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The expressions of collagen-I and HO-1 mRNAs were studied using RT-PCR. PCR reactions were performed with SYBR Green chemistry using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reactions for collagen-I and HO-1 were performed in duplicate in a 25 ml final volume containing 1X SYBR Green Master mix (Applied Biosystems) and 300 nM of primers. PCR cycling conditions were 10 min at 95ºC and 40 cycles of 20 seconds at 95ºC and 1 min at 60ºC. Data were analyzed using the absolute standard curve method and the amplification of a housekeeping gene 18S was used for normalizing

the results. The unnormalized expression of 18S mRNA did not differ between the experimental groups enabling its use as the control housekeeping gene.

4.1.3 Ventricular atrial and B-type natriuretic peptides, skeletal alpha-actin, and beta-myosin heavy chain mRNAs (study II)

Total RNA was isolated from the heart ventricles by the guanidine thiocyanate CsCl method, and 20-μg samples of RNA were transferred to nylon membranes (Osmonics) for Northern blot analysis (Lakó-Futó et al., 2003; Pikkarainen et al., 2003). Full-length rat ANP cDNA probe (a gift from Dr. Peter Davies, Queen's University, Kingston, Canada), and cDNA probes for rat BNP, skeletal alpha-actin (SkαA), and beta-myosin heavy chain (ß-MHC) and 18S were prepared. The cDNA probes were labeled, the membranes were hybridized and washed, and exposed with PhosphorImager screens (Amersham Biosciences), which were scanned with Molecular Imager FX Pro Plus and quantified using Quantity One software (Bio-Rad) as previously described (Lakó-Futó et al., 2003; Pikkarainen et al., 2003). The hybridization signals of specific mRNAs were normalized to that of 18S RNA in each sample.

4.1.4 Radioimmunoassay of 8-isoprostaglandin $F_{2\alpha}$ and11-epi-prostaglandin $F_{2\alpha}$ (studies I and III)

The determinations of 8-isoprostaglandin $F_{2\alpha}$ and 11-epi-prostaglandin $F_{2\alpha}$ were performed from urine samples collected in metabolic cages prior to and at the end of the Oxo-diet. For the measurement of urinary 8-isoprostaglandin $F_{2\alpha}$ urine samples were first vortexed and centrifuged at 3000g for 5 min. Three thousand disintegrations per minute of labeled 8-iso[^{3}H]-$PGF_{2\alpha}$ was added to 1 ml of supernatant (pH 3.0) and incubated overnight at 22ºC before extraction on a C2 silica cartridge (Applied Separation, Allentown, PA, USA). Rabbit polyclonal antibody was used at a dilution that was responsible for binding 30% of tracer. 8-iso[^{125}I]-$PGF_{2\alpha}$ methyl tyrosinate was diluted in 50 mM phosphate buffer (pH 7.4) for the use as a tracer in RIA. A series of standards was diluted in 50 mM phosphate buffer (pH 7.4) using non-labeled 8-isoprostaglandin $F_{2\alpha}$ in the concentration range of 0-5 ng/ml (0-500 pg/tube). Dextran-coated charcoal suspension was prepared using 1% neutral activated charcoal in 10 mM phosphate buffer (pH 7.4) containing 0.5% Dextran T-70. RIA was carried out in polystyrene test tubes in 50 mM

phosphate buffer (pH 7.4) containing 0.1% gelatin. The assay mixture contained 0.1 ml of antibody, 0.1 ml of 8-iso[^{125}I]-PG tracer, and 0.1 ml of 8-isoprostaglandin $F_{2\alpha}$ standards or the measured samples. The final assay volume was adjusted to 0.4 ml by the addition of buffer. Measurements were performed in duplicate. After incubation at 4ºC overnight, 0.5 ml of dextran-coated charcoal under continuous stirring was added to each assay tube except for the total count tubes to separate the bound from the free fraction. The tubes were vortexed and incubated for 10 min at 4ºC and centrifuged at 2000 g for 10 min at the same temperature. 8-iso[^{125}I]-PG$F_{2\alpha}$ radioactivity was measured from supernatant aliquots of 0.6 ml in a gamma counter (Rossi et al., 2004). The 8-isoprostaglandin $F_{2\alpha}$ concentrations were log-transformed before determination of the final values.

11-epi-prostaglandin $F_{2\alpha}$ radioimmunoassay was carried out in 50 mM phosphate buffer (pH 7.4), including 0.1% gelatin and 0.01% thiomersal (Mucha & Riutta, 2001). The assay mixture contained 0.1 ml of antiplasma, 0.1 ml of tracer and 0.1 ml of standards or the measured urine samples. The final assay volume was adjusted to 0.4 ml by the addition of assay buffer. After incubation at 41ºC overnight, dextran-coated charcoal was used for the separation of the bound from the free fraction.

4.1.5 Western blotting of renal COX-2 (study III)

Frozen kidney samples were homogenized in 400 µl of physiological salt solution made from distilled H_2O containing protease inhibitors (CompleteTM Mini EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany) using Ultra-Turrax T25 homogenizer (Janke & Kunkel GmbH & Co, IKAâ-Labortechnik, Staufen, Germany). After removal of tissue debris in centrifugation (12,000g for 15min at 4°C), protein concentrations of the supernatant protein concentrations were determined using Coomassie PlusTM Protein Assay Kit (Pierce, Rockford, IL, USA). SDS-PAGE was run on 8% resolving gel and 4% stacking gel. Subsequently, proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences UK Limited, Buckinghamshire, UK). The antibodies used were 1:250 dilution of mouse monoclonal anti-COX-2 IgG primary antibody (BD Biosciences) and 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce). Antibody binding was detected by SuperSignal West Pico chemiluminescent substrate (Pierce). The chemiluminescence signal was analyzed

with FluorChem software version 3.1. (FluorChem 8800 imaging system, Alpha Innotech Corporation, San Leandro, CA, USA).

4.1.6 Functional and morphological studies of the carotid and mesenteric arteries *in vitro* (studies I and II)

The vascular experiments were performed with 10 randomly selected rats from each group. For the studies 2 mm long standard sections of the left carotid artery, two successive 3 mm long sections from the main branch of superior mesenteric artery (beginning 3 mm distally from the mesenteric artery-aorta junction), and 1.9 mm in length second order branches from the mesenteric arterial bed were used. In the larger arteries, the force of contraction was measured with isometric force-displacement transducers and registered on a polygraph (FT 03 transducer, 7E Polygraph; Grass Instrument Co., Quincy, MA, USA) while in the small arteries the computerized Mulvany multimyograph (Model 610A, J.P. Trading, Aarhus, Denmark) was employed (P. Kööbi et al., 2004). All the arterial preparations were kept in physiological salt solution (pH 7.4) containing (mmol/l): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, and $MgSO_4$ 1.2 and aerated with 95% O_2 and 5% CO_2 at +37°C. The endothelium was left intact in the carotid arteries and in one section of the large and small mesenteric arterial rings. In the remaining mesenteric artery sections, the endothelium was removed mechanically or by perfusing air through the lumen (Jolma et al., 2003; P. Kööbi et al., 2003). The efficiency of the removal of the endothelium was confirmed by the lack of relaxation to Ach (Arvola et al., 1992).

For functional studies, the arterial preparations were suspended between hooks in an organ bath chamber in physiological salt solution and the rings were equilibrated for 1½ hours with a resting preload of 3.7 mN/mm or 4.905 mN/mm in the carotid and mesenteric arteries, respectively. The rings were allowed 30 min at baseline tension in between each concentration–response challenge. The small arterial preparations were normalized for the functional experiments so that the internal diameter of the vessel was set at 90% of that obtained when exposed to intraluminal pressure of 100 mmHg in the relaxed state (Jolma et al., 2003; P. Kööbi et al., 2003).

4.1.6.1 Arterial contractile and relaxation responses

In the carotid arteries, contraction responses to noradrenaline (NA) were cumulatively elicited, while in the large and small arterial mesenteric arterial rings, contractions were examined in response to both NA and high concentrations of K^+ (by replacing NaCl with KCl in the physiological salt solution). Endothelium-dependent relaxations to Ach, both in the absence and presence of the non-specific NO synthase (NOS) inhibitor N^G-nitro-l-arginine methyl ester (L-NAME, 0.1mmol/l), were examined in endothelium-intact carotid and mesenteric arterial (main branch) rings after precontraction with NA. Endothelium-independent relaxation responses to the NO donor sodium nitroprusside (SNP) and β-adrenoceptor agonist isoprenaline were examined in arterial rings precontracted with NA. The relaxations to the BK_{Ca}-opener NS-1619 were studied in endothelium-denuded mesenteric arterial rings precontracted with KCl (Olesen et al., 1994). The contractile responses were expressed as wall tension (mN/mm) and as percentage of maximum or as a negative logarithm of the agonist concentration producing 50% of maximal wall tension (pD_2) as appropriate, and the relaxations were depicted as percentage of pre-existing contraction.

4.1.6.2 Morphology of the small mesenteric arteries

Morphology of small mesenteric arteries at 90 mmHg intraluminal pressure was examined using a pressure myograph (Living Systems Instrumentation, Inc., Burlington, VT, USA) (Suo et al., 2002). The development of myogenic tone was inhibited by Ca^{2+}-free solution containing 30 mmol/l EDTA (Laurant et al., 1997).

4.1.7 Kidney morphology and immunohistochemistry (study III)

Five micrometer-thick kidney sections were stained with hematoxylin-eosin or periodic acid Schiff (PAS), toluidine blue, or immunohistochemistry, and processed for light microscopic evaluation by an expert blinded to the treatments.

Glomerulosclerosis (hematoxylin-eosin and PAS stain): One hundred glomeruli from each rat were examined at a magnification of ×400 and scored from 0 to 5 according to severity of the scarring (Schwarz et al., 1998): 0=normal, 1=mesangial expansion or basement membrane thickening; 2=segmental sclerosis in <25% of the tuft,

3=segmental sclerosis in 25-50% of the tuft, 4=diffuse sclerosis in >50% of the tuft, 5=diffuse glomerulosclerosis, tuft obliteration and collapse. The damage index for each rat was calculated as a mean of the scores.

Tubulointerstitial damage (hematoxylin-eosin and PAS stain): Injury consisting of tubular atrophy, dilatation, casts, interstitial inflammation, and fibrosis was assessed in 10 kidney fields at a magnification of ×100 (Schwarz et al., 1998). Damage scoring was from 0 to 4: 0=normal, 1=lesions <25% of the area, 2=lesions in 25-50% of the area, 3=lesions in >50% of the area, 4=lesions covering the whole area.

Arteriosclerosis index (PAS-stain): Arterial preparations were magnified ×100 whereafter 10 fields were randomly assigned and graded (0 to 2) according to hyaline thickening as follows: 0, no hyaline thickening; 1, mild to moderate hyaline thickening in at least one arteriole; 2, moderate or severe hyaline thickening in more than one arteriole (Racusen et al., 1999).

Kidney mast cells (toluidine blue-stain): Toluidine blue staining was applied for mast cell identification and quantification. The number of the purple-stained mast cells were counted at a magnification of ×400 and related to kidney tissue area.

Immunohistochemistry of COX-2, and smooth muscle cell alpha-actin: For the staining of COX-2 a 1:200 dilution of monoclonal anti-COX-2 IgG antibody (clone 33, BD Biosciences, San Diego, CA, USA) and for SMA a 1:200 dilution of monoclonal anti-SMA IgG antibody (code M0851, Dako Denmark A/S, Glostrup, Denmark) were used. Immunostaining was performed using the Ventana BenchMark LT Automated IHC Stainer (Ventana Medical System, AZ, USA) with the Ultra-view Universal DAB detection kit (catalog no. 760-500, Ventana Medical System) as previously described (Rantanen et al., 2014).

Tubulointerstitial COX-2 staining was scored 0 to 3: 0=no cells stained, 1=faint immunoreactivity, 2=moderate positive staining, 3=strong positive staining. Cell positivity (percentage of positive cells) was defined: 0=no cells stained, 1=1–25% positive cells, 2=26–75% positive cells, and 3=>75% positive cells. The results of both analyses were combined for the final score. In the glomeruli, the numbers of COX-2 positive cells were counted and related to tissue area. Staining of smooth muscle cell alpha-actin (SMA) was evaluated in an attempt to identify afferent arterioles from the efferent arterioles (Mazzali et al., 2002).

4.1.8 Drugs and dietary compounds

The drugs used in the study were: ketamine (Parke-Davis Scandinavia AB, Solna, Sweden), cefuroxime, diazepam (Orion Pharma Ltd., Espoo, Finland), metronidazole (B. Braun AG, Melsungen, Germany), buprenorphine (Reckitt & Colman, Hull, England), acetylcholine chloride, isoprenaline hydrochloride, N^G-nitro-L-arginine methyl ester, norepinephrine bitartrate, L-arginine, Ang II, oxonic acid (Sigma-Aldrich Chemical Co., St Louis, MO, USA), sodium nitroprusside (Fluka Chemie AG, Buchs SG, Switzerland) and NS-1619 (Sigma-Aldrich Chemical Co, St Louis, MO, USA),. The stock solutions used in the *in vitro* studies were made by dissolving the compounds in distilled water. All solutions were freshly prepared before use and protected from light.

4.1.9 Statistical analyses

For normally distributed variables, statistical analyses were carried out by using one-way and two-way analyses of variance (ANOVA), as appropriate. If the distribution of the variables was skewed, the Kruskal-Wallis test was applied. Post-hoc analyses were performed with the least significant difference test or the Mann-Whitney U-test, and the P values were corrected with the Bonferroni equation. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive observation points. Spearman's two-tailed correlation coefficient (rS) was used in the correlation analyses. The results were expressed as means and standard errors of the mean (SEM) or as medians, 25^{th} to 75^{th} percentiles, and ranges. The differences between the groups were considered significant when $P<0.05$. Unless otherwise indicated the P values in the text refer to one-way ANOVA. The statistics were performed using IBM SPSS versions 11.5, 17, and 26.0 (Armonk, New York, USA).

4.1.10 Ethics

The study design was approved by the Animal Experimentation Committee of the University of Tampere, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland (decision LSLH-2003-9718/Ym-23). The investigation conforms to the Guiding Principles for Research Involving Animals.

4.2 Subjects and methods of the hemodynamic study (study IV)

The study subjects were recruited as previously described (Kangas et al., 2019; Tahvanainen et al., 2009; Tikkakoski et al., 2013). All study participants were examined by a physician and laboratory analyses for elevated BP were taken (Williams et al., 2018). Medical and family history and lifestyle behavior were documented. Alcohol use was evaluated as standard drinks (~12 grams of absolute alcohol) per week, and smoking amount was estimated in pack-years. The exclusion criteria were known history of coronary artery disease, stroke, cardiac insufficiency, valvular heart disease, chronic kidney disease, secondary hypertension, alcohol or substance abuse, psychiatric illness other than mild to moderate depression or anxiety, heart rhythm other than sinus rhythm, and use of antihypertensive or UA lowering medications. Altogether 606 subjects, aged 20-72 years, took part in the study with each signing an informed consent. The study complies with the declaration of Helsinki and was approved by the ethics committee of the Tampere University Hospital (study code R06086M) and the Finnish Medicines Agency (Eudra-CT registration number 2006-002065-39). The study was registered in the database of clinical trials (ClinicalTrails.gov NCT01742702).

The following stable medications were used by the participants: female hormones for contraception or hormone replacement therapy (n=63), antidepressants (40), vitamin D supplements (39), hormone releasing intrauterine devices (24), thyroxin (21), inhaled glucocorticoids (17), antihistamines (16), statins (14), proton pump inhibitors (13), calcium supplementation (10), antirheumatics (6), low dose acetylsalicylic acid (6), anxiolytics (6), inhaled β_2-mimetics (4), non-steroidal anti-inflammatory drugs (3), coxibs (3), pregabalin or gabapentin (3), antiepileptics (2), varenicline (2), warfarin (2), isotretinoin (1), ezetimibe (1), and tramadol (1).

4.2.1 Laboratory measurements

Blood and urine samples were taken after about 12 hours of fasting. Plasma C-reactive protein (CRP), cystatin C, sodium, potassium, glucose, creatinine, UA, triglyceride, and total, HDL and LDL cholesterol concentrations were determined using Cobas Integra 800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland). Insulin and parathyroid hormone (PTH) were determined using electrochemiluminescence immunoassay (Cobas e411, Roche Diagnostics). Estimated GFR was calculated

using the CKD-EPI cystatin C equation (Inker et al., 2012), and insulin sensitivity was evaluated by the quantitative insulin sensitivity check index (QUICKI) (Katz et al., 2000). Plasma renin activity (GammaCoat® Plasma Renin Activity 125-I RIA Kit, DiaSorin, Saluggia, Italy) and aldosterone concentration (Active® Aldosterone RIA, Beckman Coulter, Fullerton, CA, USA) were determined using commercial kits. The Fimlab normal reference range for UA is 230-480 μmol/l for men, 155-350 μmol/l for women aged 18-49 years, and 155-400 μmol/l for women aged ≥50 years.

4.2.2 Hemodynamic measurement protocol

Hemodynamic recordings were performed by a trained nurse in a standard laboratory with a validated protocol (Koskela et al., 2013; Tahvanainen et al., 2009; Tikkakoski et al., 2013). Participants were instructed to refrain from caffeine containing products, smoking or heavy meal for ≥4 hours, and alcohol consumption for ≥24 hours prior to the investigations. The measurements were done in a supine position on the examination table with impedance cardiography electrodes placed on body surface, tonometric sensor for pulse wave analysis on left radial pulsation, and oscillometric brachial cuff for BP calibration to the right upper arm (Figure 5). The left arm with the tonometric sensor was abducted to 90 degrees in a support, which held the measurement probe at the heart level. The measurement consisted of one 5-minute period with continuous capture of hemodynamic data and for the analyses, the mean values of each 1-minute period of recording were calculated (Koskela et al., 2013; Tahvanainen et al., 2009; Tikkakoski et al., 2013).

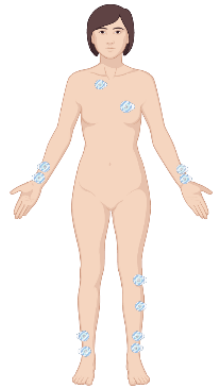

Figure 5. Placement of electrodes for the whole-body impedance cardiography signal (on the wrists, ankles, and thorax area) and for the measurement of pulse wave velocity (additional electrodes above and below the knee). Figure via BioRender by the author.

4.2.3 Pulse wave analysis

Radial BP and pulse wave were continuously recorded by a tonometric sensor (Colin BP-508T, Colin Medical Instruments Corp., USA) fixed on the radial pulse and the radial BP was calibrated approximately every 2.5 minutes by contralateral brachial BP measurements (Tahvanainen et al., 2009; Tikkakoski et al., 2013). Aortic BP and variables of wave reflection were derived with the validated SphygmoCor pulse wave monitoring system (SpygmoCor PWMx, AtCor medical, Australia) (C.-H. Chen et al., 1997). Aortic pulse pressure, augmentation index (AIx, augmented pressure/pulse pressure*100), and AIx adjusted to heart rate 75/min (AIx@75) were also determined. The central forward wave amplitude was defined as the difference between waveform foot and first systolic inflection point pressure in the aortic waveform (Kaess et al., 2012; Mitchell Gary F. et al., 2004).

4.2.4 Whole-body impedance cardiography

The changes in body electrical impedance during cardiac phases was recorded using a whole-body impedance cardiography (CircMon^{R}, JR Medical Ltd., Tallinn, Estonia). The device was used to measure heart rate, stroke volume, cardiac output, and PWV as previously described (T. Kööbi et al., 2003, 2003; T. Kööbi, Kaukinen, Ahola, et al., 1997, 1997). Systemic vascular resistance was calculated from radial BP and cardiac output measured by CircMon^{R}. Stroke volume, cardiac output and systemic vascular resistance were presented as indexes (SI, CO, SVRI, respectively) related to body surface area calculated using the DuBois equation (Du Bois & Du Bois, 1989). The method and electrode configuration have been previously reported (T. Kööbi et al., 2003; T. Kööbi, Kaukinen, Ahola, et al., 1997).

4.2.5 Statistical analyses

The data was analyzed using analysis of variance, and the Bonferroni correction was applied in the post-hoc analyses. IBM SPSS Statistics Version 26 (IBM Corporation, Armonk, NY, USA) was used for statistics. The results in the tables were presented as means and standard errors of the mean (SEM), and in the figures as means and

95% confidence intervals, and $P<0.05$ was considered significant. The hemodynamic values were calculated as averages from the minutes 3-5 of the recordings when the signal was most stable. For the analyses, the subjects were divided into quartiles of plasma UA concentration, and the quartiles were adjusted for sex; or age, sex, and body mass index (BMI), as appropriate. There were no differences between the quartiles of plasma UA in the use of the medications listed above.

Linear regression analyses with stepwise elimination were used to investigate factors independently associated with hemodynamic variables. For these analyses, the continuous variables not normally distributed were log-transformed. The covariates in the analyses were age, sex, Lg10 of BMI, alcohol consumption category, smoking status; plasma calcium, phosphate, PTH, Lg10 of triglycerides, HDL cholesterol, LDL cholesterol, Lg10 of UA, renin, aldosterone, Lg10 of CRP, sodium; Lg10 of QUICKI, eGFR, Lg10 of PWV, and mean aortic pressure, as appropriate.

5 RESULTS

5.1 Experimental animal studies (studies I, II, III)

5.1.1 Blood pressure; body, heart, and kidney weights; and markers of cardiac and volume load

At the start of the Oxo feeding, the systolic BPs and body weights were comparable in the experimental study groups in all the studies (Table 2). In study I, neither hyperuricemia nor NX influenced BP in single groups, but increased heart weight to body weight ratio was observed in both NX groups when compared with the Sham groups. In the studies II and III, the two NX groups combined displayed a significant mean systolic BP elevation when compared with the two Sham groups combined using two-way ANOVA.

Increased right and left ventricular weights and increased synthesis of heart ventricular ANP, BNP, SkαA and β-MHC mRNAs in both NX groups versus the Sham groups ($P<0.05$ for all, two-way ANOVA) indicated pressure and volume overload in experimental CKD (study II) (Figure 6). In both NX groups body weight-adjusted total kidney weight was reduced, and remnant kidney weight elevated, when compared to the Sham group (study III). Oxonic acid feeding did not influence BP, kidney or heart weights, or volume load, but it reduced final body weights (reported in studies II and III) (Table 2).

Table 2. Experimental group data in studies I, II and III that contained slightly different subgroups of the total 12 rats in each group. Oxonic acid feeding period was from week 3 to week 12.

Rats in studies I, II and III	Sham	Sham+Oxo	NX	NX+Oxo
Systolic BP at week 3 (mmHg)	120±4	121±5	127±5	125±5
Systolic BP at week 12 (mmHg)	134±7	136±6	142±6‡	152±4‡
Body weight at week 3 (g)	339±6	338±7	333±8	332±7
Body weight at week 12 (g)	433±8	412±11#	448±10	411±8†#
Heart weight				
Total (g/kg)	3.97±0.05	4.12±0.09	4.95±0.27*	5.18±0.33*
Right ventricle (g/kg)	0.28±0.01	0.29±0.01	0.34±0.03‡	0.33±0.03‡
Left ventricle (g/kg)	1.71±0.06	1.86±0.06	2.23±0.11*‡	2.41±0.18*‡
Removed kidney tissue (g/kg)			7.67±0.17	7.50±0.08
Renal tissue weight				
To body weight (g/kg)	6.4±0.2	6.0±0.1	5.2±0.3*	5.0±0.3*
Right kidney (g)	1.4±0.1	1.2±0.1	Removed	Removed
Left kidney (g)	1.4±0.1	1.2±0.1	2.3±0.1*	2.1±0.1*

Values are mean±SEM or median (25th-75th percentile), **P*<0.05 compared with the Sham group, †*P*<0.05 compared with the NX group, #P<0.05 oxonic acid treated groups versus untreated groups (two-way ANOVA), ‡*P*<0.05 NX groups compared with the Sham groups (two-way ANOVA).

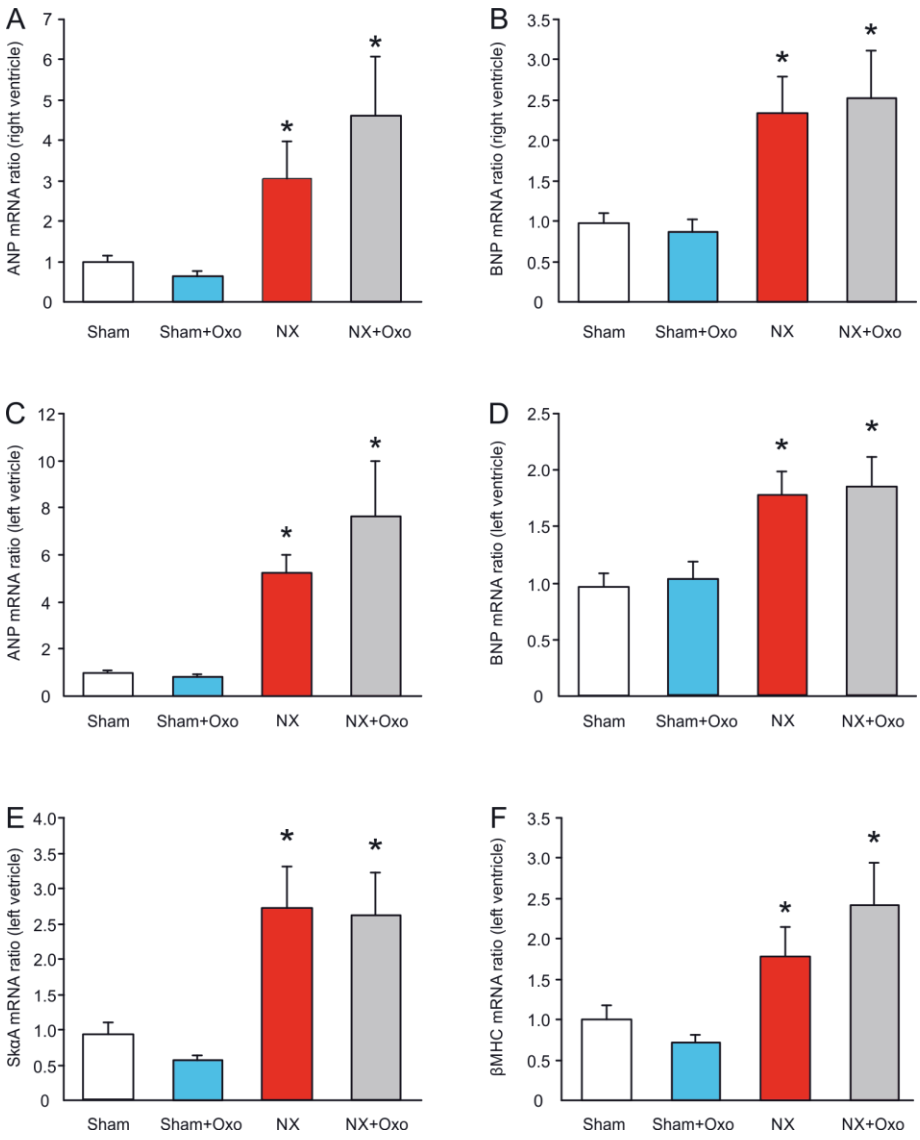

Figure 6. Right ventricular atrial natriuretic peptide (ANP) (A) and B-type natriuretic peptide (BNP) (B); and left ventricular ANP (C), BNP (D), skeletal α-actin (SkαA) (E), and β myosin heavy chain (β-MHC) (F) mRNAs in the experimental groups; values are mean±SEM, n=11 for all groups; *P<0.05, two-way ANOVA comparing both NX groups with both Sham groups.

5.1.2 Uric acid levels and biochemical measures of renal function

In studies I, II and III Oxo diet elevated plasma UA concentration approximately 2.5 to 3.5-fold in comparison with the respective control groups (Table 3).

Table 3. Plasma uric acid, plasma chemistry, and pH.

Rats in studies I, II and III	Sham	Sham+Oxo	NX	NX+Oxo
Uric acid week 5 (µmol/l)	50.3±13.0	106.8±15.5*	49.1±10.2	86.6±9.6*
Uric acid week 12 (µmol/l)	36±11	117±21*	63±19	152±19*†
Creatinine (µmol/l)	40.2±5.1	48.5±3.0	81.9±3.1*	83.1±8.1*
Creatinine clearance (ml/min)	2.9±0.4	2.0±0.2*	1.2±0.1*	1.2±0.1*
Urea (mmol/l)	6.6±0.3	8.3±0.4	13.5±0.9*	14.5±2.0*
TRAP (µmol/l)	422±52	611±70*	491±70	714±60*†
Blood pH	7.42±0.03	7.37±0.02	7.34±0.03	7.37±0.02
Renin activity (ng/ml/h)	27.3 (22.8-30.9)	31.2 (27.8-41.4)	2.0 (0.3-3.1)*	5.0 (2.7-9.9)*
Phosphate (mmol/l)	1.4±0.1	1.5±0.1	1.9±0.2‡	1.9±0.2‡
Calcium (mmol/l)	2.42±0.02	2.36±0.02	2.42±0.04	2.42±0.02
Sodium (mmol/l)	136.5±0.5	137.3±0.6	136.7±0.9	137.0±0.5
Potassium (mmol/l)	4.1±0.1	3.8±0.1	4.3±0.2	4.4±0.2

Values are mean±SEM or median (25^{th}-75^{th} percentile), *$P<0.05$ compared with the Sham group, †$P<0.05$ compared with the NX group, ‡$P<0.05$ NX groups compared with the Sham groups (two-way ANOVA).

The NX operation elevated plasma creatinine and urea concentrations approximately 2-fold, the concentrations of which were not affected by hyperuricemia. Plasma UA levels were nearly doubled in the NX group vs. the Sham group, but the increase was not statistically significant (Table 3). Creatinine clearance (study III) was reduced in all groups when compared with the Sham group. Plasma phosphate was elevated in both NX groups versus the Sham groups, but the concentrations of calcium, sodium, and potassium did not differ between the experimental groups. Neither UA nor NX influenced blood pH (study I) (Table 3).

5.1.3 Urinary and renal oxidative stress markers and plasma TRAP

At start of the Oxo diet, the 24-h urinary excretion of 8-isoprostaglandin $F_{2\alpha}$ was similar in all groups, while at the end of the study the excretion was markedly increased in the NX group (study I) (Figures 7A and 7B). At the end of the study, 8-isoprostaglandin $F_{2\alpha}$ excretion was lower in the Sham+Oxo and NX+Oxo groups than in the respective controls (Figure 7B). Oxonic acid feeding caused a 1.5-fold increase in the plasma concentration of TRAP in Sham+Oxo and NX+Oxo rats when compared with the Sham and NX groups, respectively (Table 3). There was a marked correlation between plasma UA and TRAP (r=0.910, P=0.000), while plasma UA also correlated with maximal relaxation to Ach in the NX and NX+Oxo groups (Figure 7C and D).

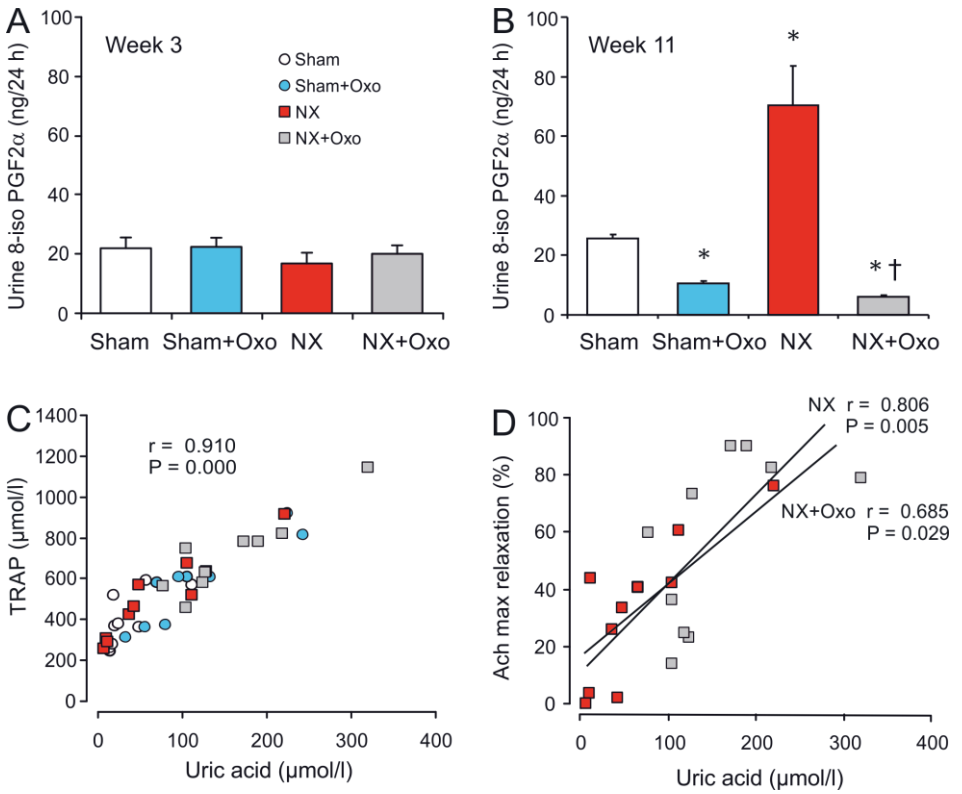

Figure 7. 24-h urinary excretion of 8-isoprostaglandin F2α (8-iso-PGF2α) at the start (A) and at the end (B) of the 9 week 2.0% oxonic acid feeding. Scatter plots of plasma uric acid and (C) total peroxyl radical trapping capacity (TRAP), and (D) maximal relaxation to Ach in vitro in the study groups; *P<0.05 vs. the Sham group, †P<0.05 vs. the NX group.

Urinary excretion of 11-epi-prostaglandin $F_{2\alpha}$ was higher in both NX groups than in the Sham groups at start of the Oxo diet (study III) (Figure 8A). The excretion was increased in the Sham and NX groups at the end of the study (Figure 8B). However, in the Sham+Oxo and NX+Oxo rats the excretion was approximately 75% lower than in the respective control groups (Figure 8B). Kidney HO-1 mRNA content was higher in the NX and NX+Oxo groups than in the Sham group, but UA elevation did not influence renal HO-1 mRNA expression (Figure 8C). The number of mast cells in the kidney tissue was elevated following subtotal nephrectomy but their number was significantly lower in the NX+Oxo rats than in the NX rats (Figure 8D). There was a significant correlation between the kidney mast cell count and 24-hour urinary 11-epi-prostaglandin $F_{2\alpha}$ excretion (r_S=0.415, P=0.003).

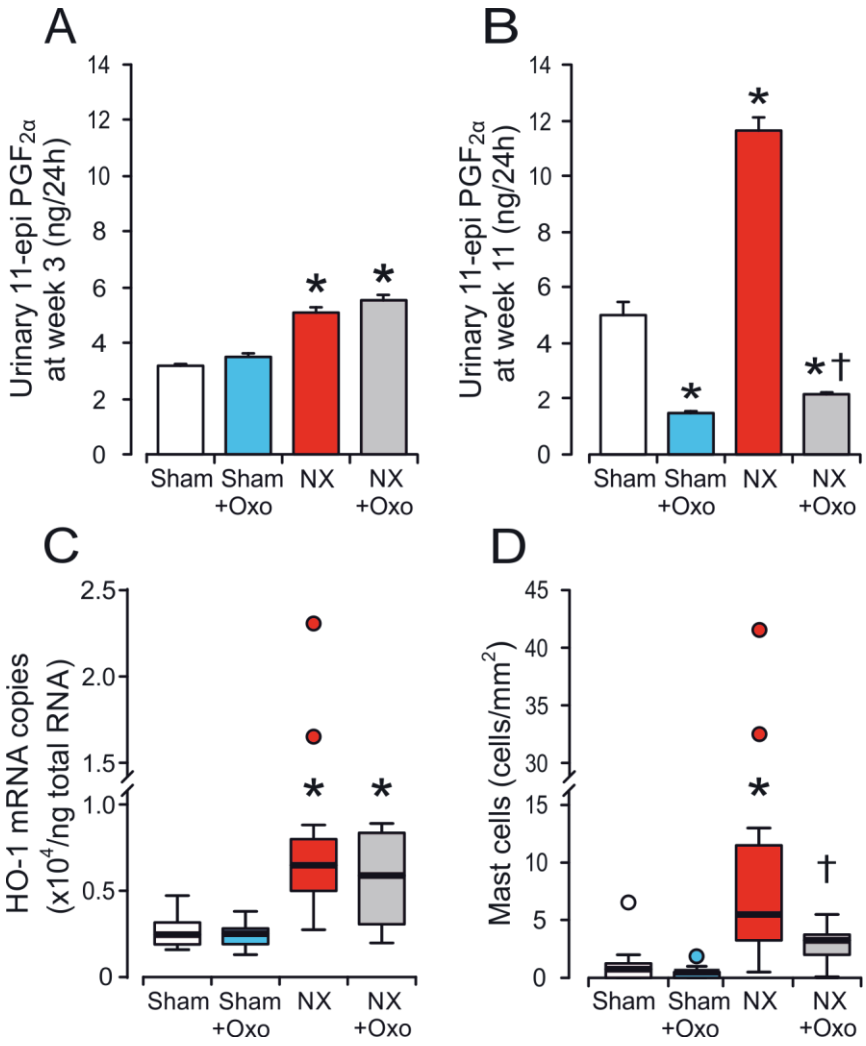

Figure 8. The 24-hour urinary excretion of 11-epi-prostaglandin F2α during study week 3 (A) and study week 11 (B), kidney hemooxygenase-1 (HO-1) mRNA expression (C), and kidney mast cell content (D) in the study groups (n=12 in each group).

5.1.4 Functional and structural studies of the carotid and mesenteric arteries

5.1.4.1 Functional responses of the carotid artery *in vitro*

Vasorelaxations to Ach in the endothelium-intact carotid arteries were reduced in both NX groups when compared with the Sham groups, but the maximal response to Ach was improved in the NX+Oxo rats when compared with the NX group (Figure 9A). Experimental hyperuricemia did not influence on Ach-induced vasorelaxation in the Sham rats. Inhibiting NOS with L-NAME abolished the relaxation to Ach in all groups, indicating that the relaxation response to Ach was mainly mediated via NO (Figure 9B). The relaxation responses to nitroprusside were reduced in both NX groups when compared with the Sham and Sham+Oxo groups, but the reduction was less marked in the NX+Oxo group (Figure 9C). Higher relaxation to 10 nmol/l nitroprusside was observed in the NX+Oxo group than in the NX group (P=0.027) (Figure 9C). Vasorelaxations elicited by isoprenaline were similar in all groups (Figure 9D). All study groups showed similar contractile responses to NA (study I).

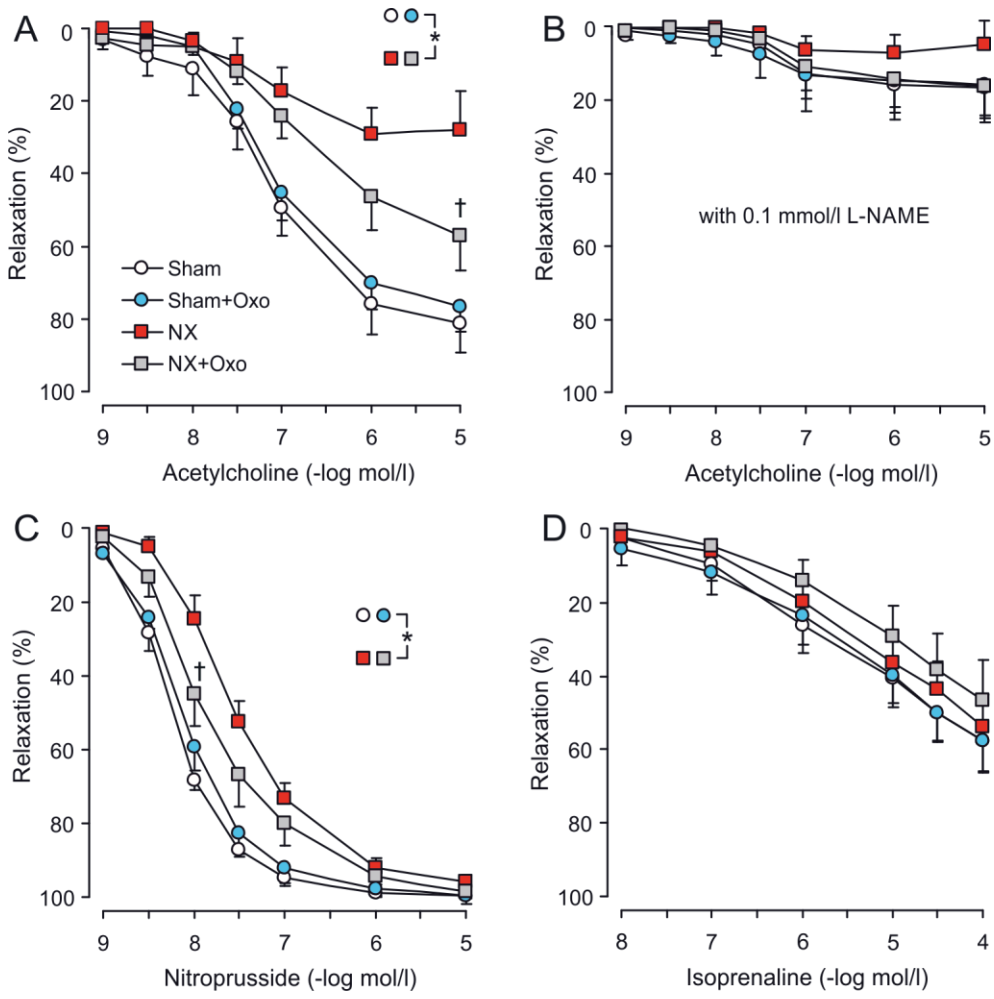

Figure 9. Carotid artery relaxation in vitro in arterial rings precontracted with 1 µmol/l noradrenaline. Responses to acetylcholine without (A) and with L-NAME (B), and nitroprusside (C) and isoprenaline (D); *P<0.05, ANOVA for repeated measurements, †P<0.05 compared with the corresponding individual concentration in the NX group.

5.1.4.2 Functional responses of the mesenteric arteries *in vitro*

In the main branch of the mesenteric artery, there was a slightly higher contractile sensitivity to NA in the NX groups than in the two Sham groups (P<0.001, two-way ANOVA). However, the relaxations were examined after 5 µM NA induced precontraction that induced approximately 70-80% of the maximal response in all groups, and therefore the differences in the contractions to NA did not curtail the

results on vasorelaxation. The contractile responses to KCl were similar in all groups (Table 4). In the small arterial rings, maximal wall tension in response to NA was higher in the NX rats than in Sham rats (P=0.03, two-way ANOVA), while the sensitivity to NA (pD_2) was similar in all groups. Responses to KCl were comparable between NX and Sham rats also in the small mesenteric artery (Table 4).

Table 4. Parameters of contractile responses of isolated mesenteric arterial rings (main and second order branches). Values are mean ±SEM, n=10 for all groups. pD_2 is the negative logarithm of the concentration of agonist producing 50% of the maximal response. ‡P<0.05 NX groups compared with Sham groups using two-way ANOVA.

Main branch	**Sham**	**Sham+Oxo**	**NX**	**NX+Oxo**
Noradrenaline				
pD_2 (−log mol/l)	6.26±0.14	5.97±0.05	6.49±0.13‡	6.64±0.11‡
Maximal wall tension (mN/mm)	7.30±0.49	8.60±049	9.14±0.63	9.64±1.52
KCl				
pD_2 (−log mol/l)	1.51±0.02	1.51±0.03	1.51±0.03	1.53±0.03
Maximal wall tension (mN/mm)	6.79±0.85	7.01±0.94	7.53±0.67	8.02±0.96
Small artery				
Noradrenaline				
pD_2 (−log mol/l)	5.81±0.08	6.00±0.09	5.81±0.09	5.87±0.11
Maximal wall tension (mN/mm)	5.46±0.17	5.82±0.50	6.63±0.54‡	6.58±0.39‡
KCl				
pD_2 (−log mol/l)	1.41±0.01	1.42±0.02	1.39±0.02	1.39±0.03
Maximal wall tension (mN/mm)	5.59±0.43	5.93±0.56	6.37±0.49	6.84±0.32

The relaxation responses induced by Ach were impaired in both NX groups when compared with the Sham groups, but in the mesenteric artery hyperuricemia did not influence on Ach-elicited relaxations (P=0.208 NX versus NX+Oxo groups, ANOVA for repeated measurements) (Figure 10A). The presence of L-NAME equally reduced the relaxation to Ach in both Sham groups, while in the NX and NX+Oxo groups the response was almost abolished (Figure 10B). Vasorelaxation mediated via cGMP in smooth muscle cells, as indicated by reduced relaxation to SNP, was impaired in NX and NX+Oxo groups when compared with the Sham and Sham+Oxo groups (P=0.008, two-way ANOVA for repeated measurements) (Figure 10C). The effect of Oxo feeding was observed only in relaxation responses elicited by BK_{Ca} opener NS-1619, where in the NX+Oxo group the response was

reduced when compared with all other groups (P=0.032, ANOVA for repeated measurements) (Figure 10D).

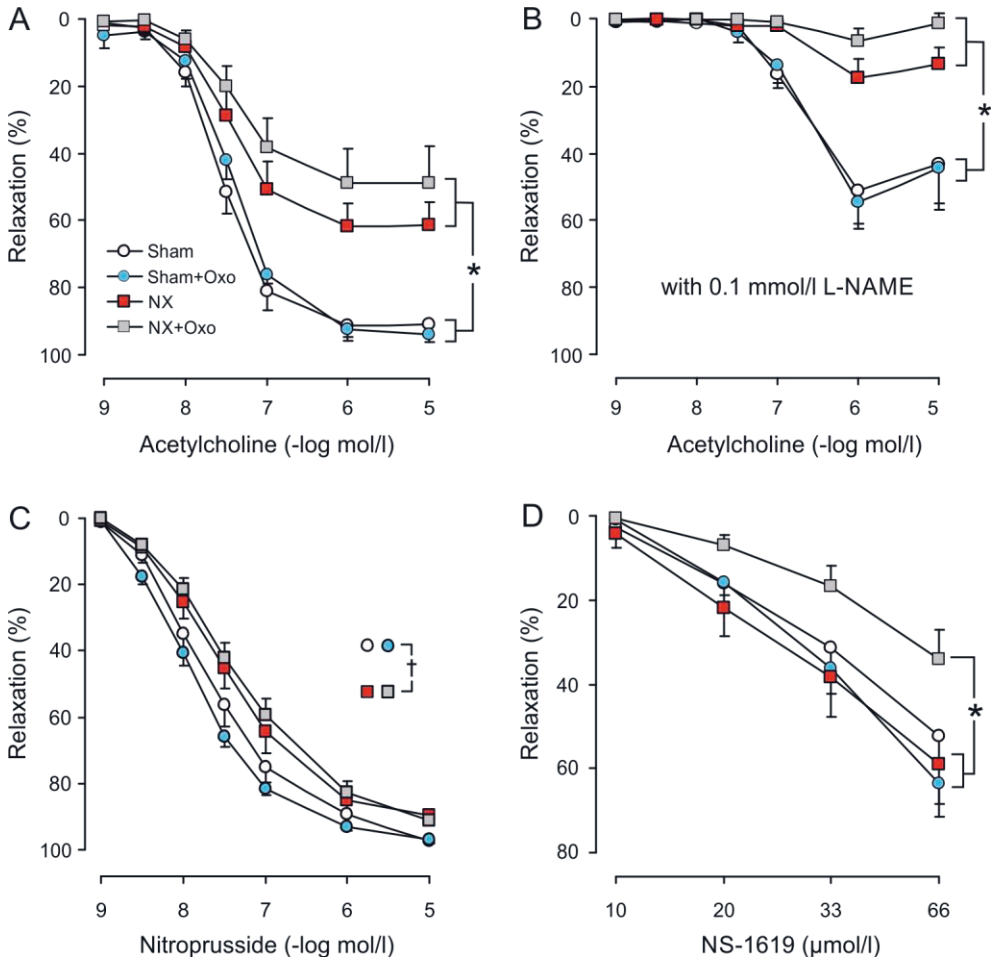

Figure 10. Mesenteric artery relaxation *in vitro* in arterial rings precontracted with noradrenaline (A and B) or KCl (C and D). Responses to acetylcholine (without and with L-NAME), nitroprusside, and NS-1619; *P<0.05, ANOVA for repeated measurements, †P<0.05 compared with the corresponding individual concentration in the NX group.

5.1.4.3 Morphology of the small mesenteric artery *in vitro*

When compared to the Sham rats, arterial wall of the NX rats exhibited hypertrophic remodeling: increased wall thickness, wall to lumen ratio, and wall cross-sectional

area without alteration in lumen diameter (Figure 11). Experimental hyperuricemia had no effect on the structure of the small mesenteric artery.

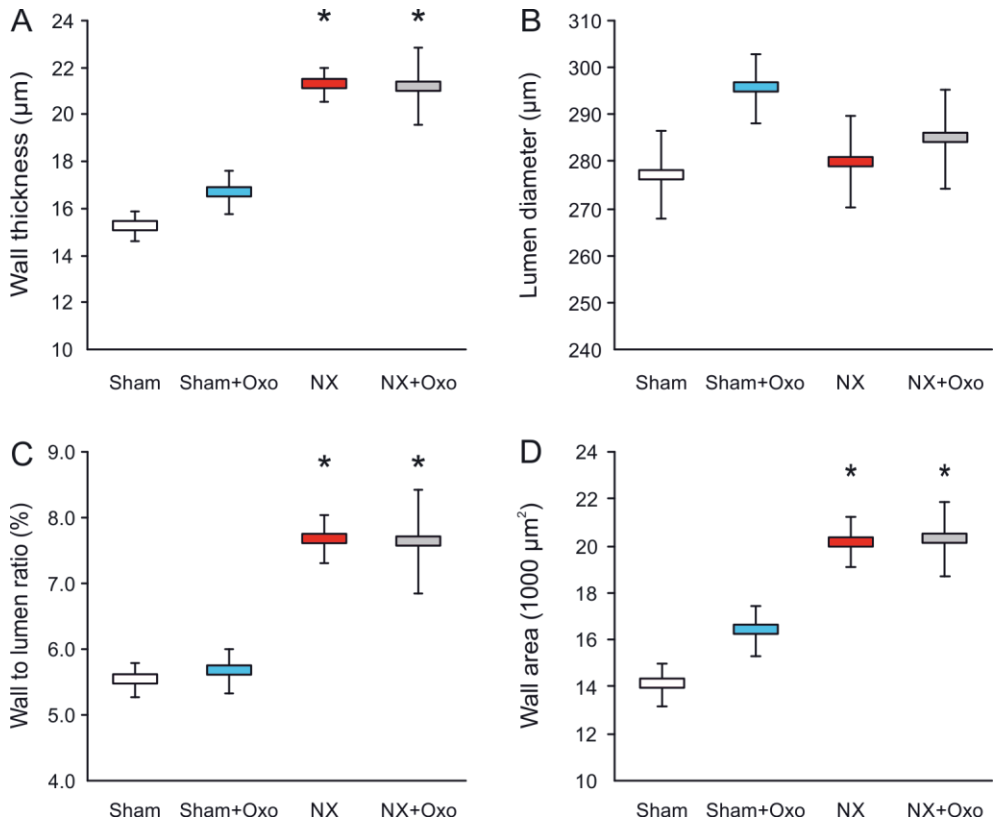

Figure 11. Arterial morphology. Small artery wall thickness (µm) (A), lumen diameter (µm) (B), wall to lumen ratio (%) (C), and wall area (D) at 90 mmHg; values are mean±SEM, n=10 for all groups; *P<0.05 versus Sham.

5.1.5 Renal histology

Proteinuria and indices of histological scarring, i.e. arteriosclerosis, glomerulosclerosis and tubulointerstitial damage, were significantly increased in the NX group, while all these indices were alleviated in the NX+Oxo group when compared with the NX group (Figure 12A-D). There was also a significant correlation between 24-hour urinary protein excretion and the glomerulosclerosis score (R=0.887) (Figure 12E). A clear staining for smooth muscle α-actin in the glomerular afferent and efferent arteries adjacent to the glomeruli was observed, but

with the present techniques we could not reliably differentiate the afferent arterioles from the efferent arterioles (Figure 12F). Therefore, further analyses of the preglomerular arterioles were not performed.

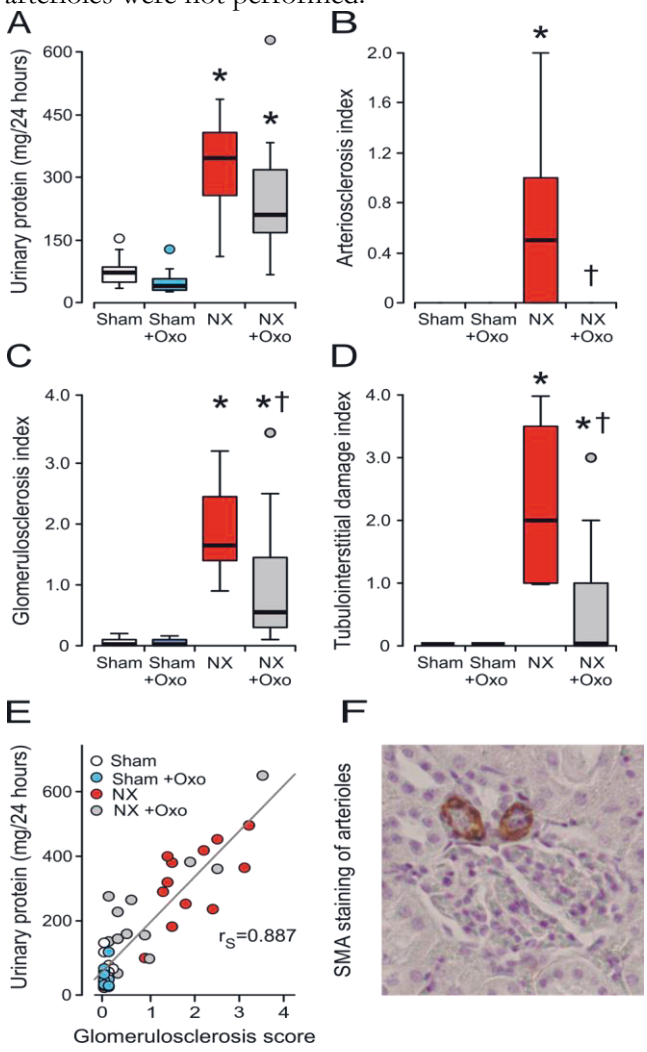

Figure 12. Urinary protein excretion during study week 12 (A), kidney arteriosclerosis index (B), glomerulosclerosis index (C), correlation between urinary protein excretion and glomerulosclerosis score (D), tubulointerstitial damage index (E), and representative photomicrograph of smooth muscle α-actin (SMA) staining of the glomerular arterioles (F) in the study groups (n=11-12 in each group). Values are median (thick line), 25th-75th percentile (box), and range (whiskers), outliers depicted as small circles; *P<0.05 versus Sham, †P<0.05 versus NX.

5.1.6 Renal markers of inflammation and collagen I mRNA

In the kidney sections, a clear staining of glomerular and tubulointerstitial COX-2 was observed (Figures 13A and B). When analyzed using Western blotting, lower COX-2 content was observed in the Sham-Oxo and NX-Oxo rats than in the rats ingesting normal diet ($P=0.046$, two-way ANOVA, Figure 13C). In immunohistochemistry analyses, tubulointerstitial COX-2 score was lower in the Sham+Oxo group when compared with the Sham group, and in the NX+Oxo group versus the NX group (Figure 13D). The COX-2 staining was abundant in tubuli with a thick epithelium corresponding to the ascending limb of the loop of Henle (Harris & Breyer, 2001). In the glomeruli, the number of COX-2 positive cells was lower in the Sham+Oxo group than in the Sham group, while the glomeruli of the NX groups had the lowest number of COX-2 positive cells (Figure 13E). The 5/6 NX model presented with increased kidney tissue collagen I mRNA expression ($P=0.022$, two-way ANOVA), but collagen I mRNA expression was not influenced by the Oxo diet (Figure 13F).

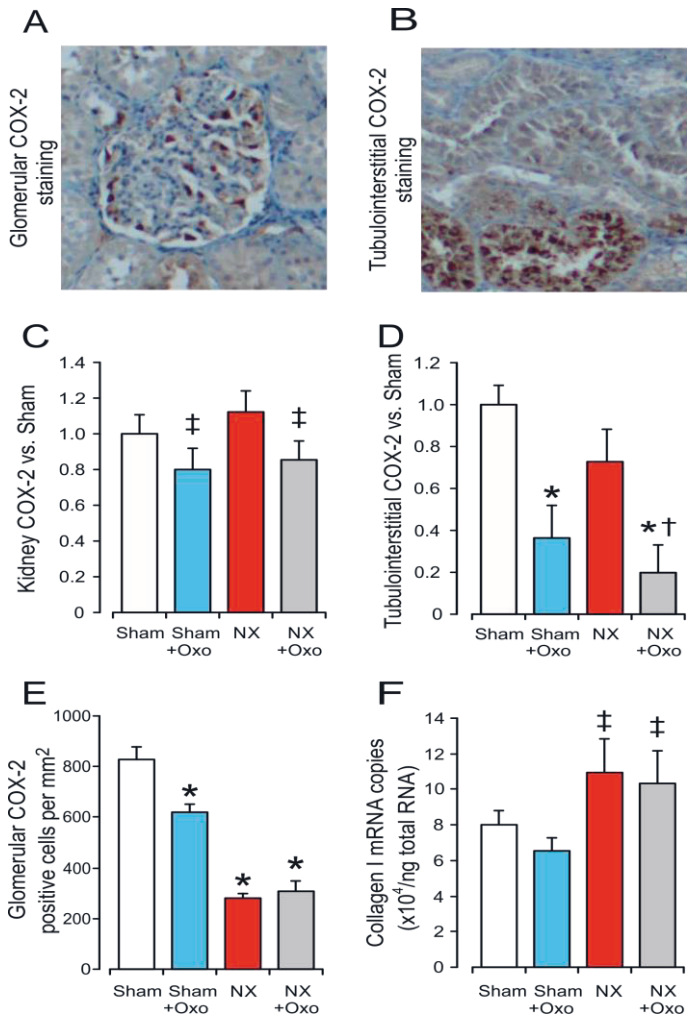

Figure 13. Photomicrographs of the immunohistochemical staining of glomerular (A) and tubulointerstitial (B) cyclooxygenase-2 (COX-2), Western blotting of kidney tissue COX-2 content (C), tubulointerstitial COX-2 score (D), number of COX-2 positive cells in the glomeruli (E), and collagen I mRNA expression (F) in the study groups (n=9-11 in each group). Groups as in Figure 1, values are mean ± SEM; *P<0.05 versus Sham, †P<0.05 versus NX, ‡P<0.05 oxonic acid diet versus normal diet (C), and ‡P<0.05 NX groups versus the Sham groups (F) using two-way ANOVA.

5.2 Plasma uric acid concentration and hemodynamic variables in healthy normotensive and never-treated hypertensive subjects (study IV)

5.2.1 Study subjects and laboratory determinants

The mean plasma UA concentrations were (258±3 μmol/l) in women and (344±4 μmol/l) in men (P<0.001), and the subjects in the highest plasma UA quartile (Q4) were significantly older than the subjects in the lowest quartile (Q1). The weight and BMI were higher in plasma UA quartiles Q3 and Q4 versus Q1 and Q2, while the heights were similar in all groups (Table 5).

Table 5. Basic characteristics of the study participants in sex adjusted quartiles from lowest (Q1) to highest (Q4) concentrations of fasting plasma uric acid.

	Q1 N=141	Q2 N=155	Q3 N=165	Q4 N=144
Male/female (n)	74/77	73/79	76/77	72/78
Age (years)	43.7(0.96)	44.3(0.95)	44.3(0.95)	47.5(0.96)*
Weight (kg)	74.9(1.2)	77.5(1.2)	82.1(1.2)*†	86.5(1.2)*†
Height (cm)	173.0(0.8)	172.9(0.8)	173.2(0.8)	172.8(0.8)
Body mass index (kg/m^2)	24.9(0.33)	25.7(0.33)	27.3(0.32)*†	28.9(0.33)*†‡

Results shown as mean (standard error of mean); *P < 0.05 versus Q1; †P < 0.05 versus Q2; ‡P < 0.05 versus Q3

Plasma UA quartiles were adjusted for age, sex and BMI with the mean plasma UA levels being 234, 278, 314 and 373 μmol/l in the respective groups. Office systolic BP was higher in Q4 than Q1, while Q3 and Q4 had significantly higher office diastolic BP in comparison with Q1 (Table 6). The proportion of subjects with office hypertension >140/90 mmHg was higher in Q3 than Q1, while the office heart rate, percentage of smokers and average alcohol intake were corresponding in all quartiles. Plasma CRP was slightly higher in Q4 when compared to Q1 and Q2, and QUICKI was lover in Q4 than Q1. Plasma triglyceride concentration was higher in Q3 and Q4 when compared with Q1, and in Q4 versus Q2, and LDL cholesterol was higher in Q3 than in Q1. Other laboratory determinants from the blood and urine did not differ between the groups (Table 6).

Table 6. Clinical characteristics and laboratory results of the study participants in age, sex and body mass index adjusted quartiles of fasting plasma uric acid concentrations.

	Q1 N=141	Q2 N=155	Q3 N=165	Q4 N=144
Office measurements				
Systolic BP (mmHg)	136 (1.5)	140 (1.5)	142 (1.5)	143 (1.5)*
Diastolic BP (mmHg)	87 (0.9)	89 (0.9)	90 (0.9)*	91 (0.9)*
Heart rate (bpm)	66.5 (0.8)	67.2 (0.8)	67.7 (0.8)	68.3 (0.9)
Number (%) of participants with BP ≥140/90 mmHg	68 (48.2)	92 (59.4)	106 (64.2)*	89 (61.8)
Current Smokers (number)	20	18	18	19
Alcohol (standard drinks/week)	2 [0-6]	2 [0-5]	3 [1-5]	3 [1-7]
Uric acid (µmol/l)	234 (4)	278 (4)*	314 (4)*†	373 (4)*†‡
Sodium (mmol/l)	140 (0.2)	141 (0.2)	140 (0.2)	140 (0.2)
Potassium (mmol/l)	3.8 (0.0)	3.8 (0.0)	3.8 (0.0)	3.8 (0.0)
Calcium (mmol/l)	2.29 (0.01)	2.30 (0.01)	2.31 (0.01)	2.32 (0.01)
Phosphate (mmol/l)	0.95 (0.01)	0.97 (0.01)	0.96 (0.01)	0.99 (0.01)
C-reactive protein (mg/l)	0.5 [0.5-1.4]	0.8 [0.5-1.8]	1.0 [0.5-2.1]	1.0 [0.5-2.1]*†
Renin activity (ng Ang I/ml/h)	0.6 [0.3-1.1]	0.7 [0.4-1.2]	0.7 [0.5-1.3]	0.8 [0.5-1.3]
Aldosterone (pmol/l)	422 [292-569]	449 [329-609]	423 [320-572]	461 [338-620]
PTH (pmol/l)	4.32 (0.13)	4.51 (0.13)	4.75 (0.13)	4.66 (0.14)
eGFR (ml/min/1.73m^2)	102 (1.2)	100 (1.2)	97 (1.2)	98 (1.2)
Albumin excretion (µg/min)#	4 [3-5]	4 [3-6]	4 [3-5]	4 [3-5]
Total cholesterol (mmol/l)	5.0 (0.1)	5.0 (0.1)	5.2 (0.1)	5.2 (0.1)
Triglycerides (mmol/l)	0.9 [0.6-1.2]	1.0 [0.7-1.3]	1.1 [0.8-1.5]*	1.2 [0.8-1.8]*†
HDL cholesterol (mmol/l)	1.65 (0.03)	1.56 (0.03)	1.55 (0.03)	1.57 (0.03)
LDL cholesterol (mmol/l)	2.9 (0.1)	3.0 (0.1)	3.2 (0.1)*	3.1 (0.1)
Glucose (mmol/l)	5.4 (0.1)	5.4 (0.1)	5.4 (0.1)	5.5 (0.1)
Insulin (mU/l)	7.2 (1.4)	7.9 (1.4)	10.7 (1.4)	9.8 (1.5)
QUICKI	0.361 [0.342-0.381]	0.359 [0.367-0.376]	0.352 [0.332-0.372]	0.345 [0.324-0.373]*

Results shown as mean (standard error of mean) or median [27th-75th percentile]; PTH, parathyroid hormone; eGFR, cystatin C-based estimated glomerular filtration rate (CKD-EPI); HDL, high density lipoprotein; LDL; low density lipoprotein; QUICKI, quantitative insulin sensitivity check index; #albumin excretion results were available 114-130 subjects in each quartile; *P<0.05 vs. Q1; †P<0.05 vs. Q2; ‡P<0.05 vs. Q3.

5.2.2 Hemodynamic measurements

Radial and aortic BPs were comparable in plasma UA quartiles adjusted for age, sex, and BMI (Figure 14A-D).

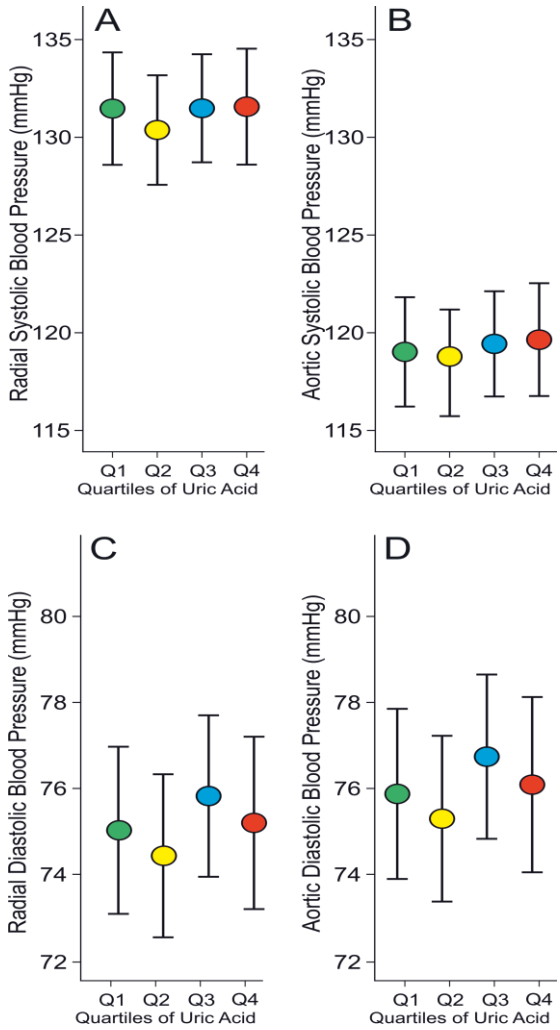

Figure 14. Radial (*A*) and aortic (B) systolic blood pressure, and radial (C) and aortic (D) diastolic blood pressure in 606 subjects presented in age, body mass index, sex, and plasma C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean.

Aortic pulse pressure, forward wave amplitude and augmentation index were similar in all quartiles, but aortic to popliteal PWV was higher in Q4 versus Q3 and Q1 (Figures 15A-D). When analyzed separately in men and women, PWV was higher in Q4 versus Q1 in men, while in women no significant differences in PWV between the plasma UA quartiles were observed (Figure 15E and F). The Pearson correlation between plasma UA concentration and PWV was 0.351 among all study subjects ($P<0.001$), 0.338 in women ($P<0.001$), and 0.242 in men ($P<0.001$).

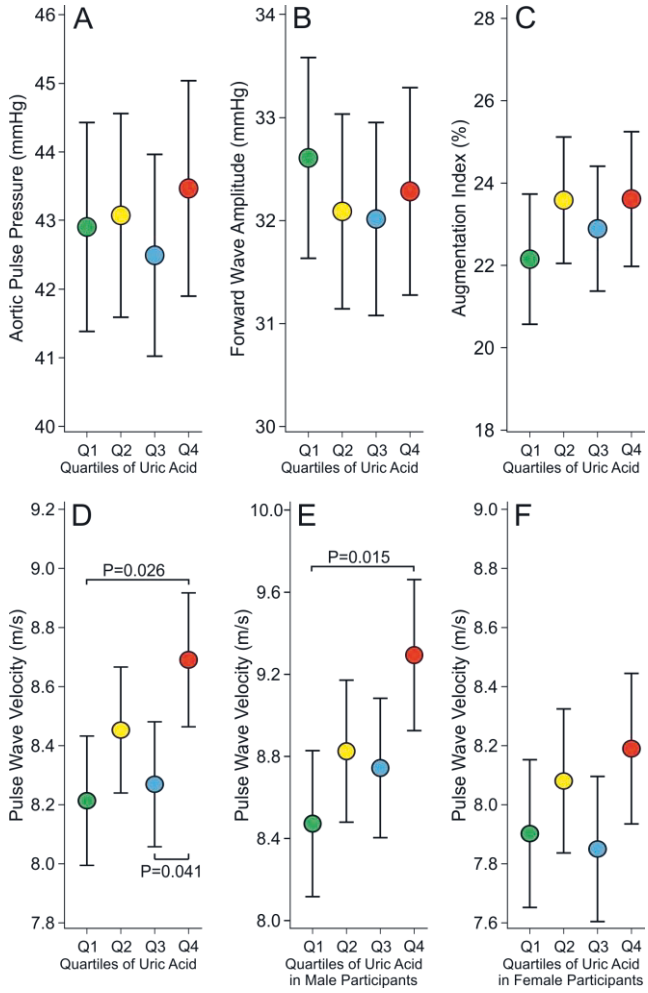

Figure 15. Aortic pulse pressure (A), forward wave amplitude (B), augmentation index (C), and pulse wave velocity (D) in 606 all subjects, and pulse wave velocity separately in men (E) and women (F), presented in age, body mass index, sex, C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean.

Heart rate, stroke volume, cardiac output and systemic vascular resistance did not differ between groups. (Figure 16).

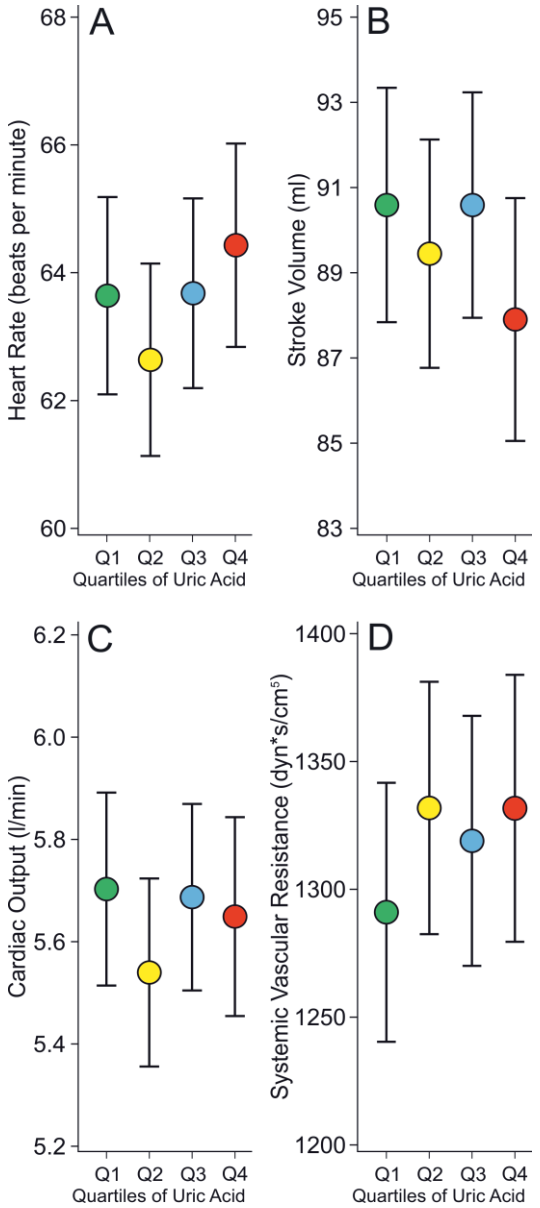

Figure 16. Heart rate (A), stroke volume (B), cardiac output (C) and systemic vascular resistance (D) in 606 subjects presented in age, body mass index, sex, C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean.

5.2.3 Hemodynamic variables in linear regression analyses

In the linear regression analyses PWV, eGFR, plasma concentration of calcium and PTH, and QUICKI were independent explanatory factors for aortic systolic ($R^2 = 0.399$) and diastolic ($R^2 = 0.350$) BPs. Systolic BP was independently associated with LDL cholesterol and triglycerides, while diastolic BP associated with male sex and high alcohol consumption.

The independent explanatory factors for PWV were age, ejection duration, mean aortic pressure, plasma UA, plasma triglycerides, BMI, and current smoking ($R^2=0.591$) (Table 7). When the subjects with plasma UA≥370 μmol/l were excluded from the regression analysis, plasma UA concentration was still associated with PWV in the remaining 507 participants (beta=0.003, P=0.006). Furthermore, for every 100 μmol/l increase in plasma UA, the associated increase in PWV was 0.9 m/s. When the explanatory variables for PWV were analyzed separately for both sexes, in women, the explanatory variables for PWV were age, mean aortic pressure, heart rate, plasma triglycerides, and plasma UA ($R^2=0.580$), and in men age, ejection duration, plasma UA, mean aortic pressure, BMI, and LDL cholesterol ($R^2=0.532$). In additional regression analyses, plasma UA was not an explanatory factor for forward wave amplitude, augmentation index, systemic vascular resistance, stroke volume, heart rate, or cardiac output.

Table 7. Significant explanatory variables for aortic to popliteal pulse wave velocity in linear regression analysis with stepwise elimination.

Pulse wave velocity (m/s)	B	Beta	P
Men and women, $R^2=0.591$			
(constant)	7.594		< 0.001
Age	0.076	0.501	< 0.001
Ejection duration	-0.020	-0.223	< 0.001
Mean aortic pressure	0.022	0.180	< 0.001
Uric acid	0.004	0.168	< 0.001
Body mass index	0.032	0.077	0.019
Lg10 of triglycerides	0.557	0.069	0.034
Current smoking	-0.363	-0.067	0.015

Variables in Model: Age, sex, body mass index, alcohol consumption category (low, moderate, high), smoking status (current, previous), mean aortic pressure, heart rate, ejection duration, eGFR, uric acid, HDL cholesterol,

LDL cholesterol; Lg_{10} of triglycerides; Lg_{10} of C-reactive protein, and QUICKI. HDL high-density lipoprotein, LDL low-density lipoprotein, QUICKI quantitative insulin sensitivity check index, eGFR estimated glomerular filtration rate from plasma cystatin-C using the CKD-EPI formula.

6 DISCUSSION

For long, UA was considered as an inert metabolic by-product of purine metabolism, with gout being the only clinically relevant manifestation of hyperuricemia. In recent decades, however, increasing epidemiological evidence has linked high UA levels to a range of chronic cardiovascular and renal pathologies, possibly via a mechanism involving increased oxidative stress. While the previous experimental studies have mainly focused on the effects of hyperuricemia in the kidney, less in known about the systemic cardiovascular effects of UA. As cardiovascular complications are the leading cause of morbidity and mortality in CKD, exploring the effects of UA also in the systemic vasculature is of importance.

Cardiovascular disease risk in hyperuricemic subjects has been coupled to increased large arterial stiffness, as indicated by elevated PWV. Increased PWV, along with other sub-clinical hemodynamic alterations, can be observed before BP elevation is detected by the standard clinical methods, emphasizing the potential benefits of using these hemodynamic parameters in individual cardiovascular risk evaluation. Increasing the knowledge over the potential effects of UA on human hemodynamics will help to understand the mechanisms via which UA could mediate its actions in the vasculature. Ultimately this could help to guide the targeting of lifestyle and pharmacological interventions more individually in a timely manner.

6.1 Study design and methods

6.1.1 Experimental model of chronic renal insufficiency and oxonic acid-induced hyperuricemia (studies I, II, III)

In one of the first experimental studies exploring the Oxo model of hyperuricemia in CKD, renal disease was induced in the rat with dietary calcineurin inhibitor cyclosporine (Mazzali, Kim, et al., 2001). In this model, renal abnormalities associated with hyperuricemia constituted mainly of arterial hyalinosis and

tubulointerstitial damage. Notably, renal changes developed only if accompanied by a very low salt diet. Later, most of the experimental UA studies using a rodent model of CKD have employed the 5/6 NX model in the rat. This is the most common model of experimental CKD, as it adequately mimics many features of human CKD, including progressive decrease in GFR, hypertension, albuminuria, glomerulosclerosis, and oxidative stress (Morrison & Howard, 1966). In the 5/6 NX model applied in the previous UA studies, renal insufficiency has been established by removing the whole right kidney, and a by a selective ligation of the contralateral renal artery branches (Kang, 2002; Sanchez-Lozada et al., 2005). In the present studies I-III, renal artery ligation was replaced by the resection of the upper and lower poles of the left kidney, also a widely used model of experimental CKD (Jolma et al., 2003). The 5/6 NX ligation method results in higher BP and more severe renal injury, probably due to up-regulation of RAAS in the peri-infarct zone vascularized by the ligated arteries, whereas the surgical 5/6 NX model is characterized by low plasma renin (Eräranta et al., 2008; Griffin & Bidani, 1994; Pörsti et al., 2004). In the current studies, the rats were randomized to study groups so that they had similar BPs, daily urine volumes, and body weights after a three-week-long post-operative recovery period. This differs from previous experiments, where the randomization has been done immediately after the operation. Randomization without the recovery period probably increases the risk for selection bias, as the chronic compensatory changes take several weeks to develop and are not as pronounced in the early course than in later phase of the renal disease (Shirley & Walter, 1991).

In all three experimental studies, the NX rats featured several biochemical and metabolic characteristics of moderate renal insufficiency: increased plasma creatinine, reduced creatinine clearance, proteinuria, and increased renal and systemic oxidative stress. There was a clear trend toward higher circulating UA levels in the NX rats, but the difference was not statistically significant. Furthermore, the kidneys of the NX rats featured advanced renal histological scarring: glomerulosclerosis, arteriosclerosis, and tubulointerstitial damage, findings that were accompanied by increased kidney mast cell infiltration. Increased COX-2 and HO-1 mRNA indicated inflammatory and pro-oxidative state in the kidney. Impaired endothelium-dependent vasorelaxation in the conduit-size arteries and hypertrophic remodeling of the resistance mesenteric arteries are all previously described characteristics of stage 2-3 renal insufficiency (Jolma et al., 2003; P. Kööbi et al., 2003). During the 12 weeks of total follow-up time, CKD was associated with only a mild systolic BP elevation, while increased heart ventricle weights and ventricular

natriuretic peptides indicated permanent volume load in CKD. This is concordant with the previous studies with 20 weeks of follow-up showing a significant elevation in systolic BP later in the disease progression (P. Kööbi et al., 2006). It should be noticed that while BP measurement with the non-invasive tail-cuff method is not as accurate as the "gold-standard" telemetry, in proper use the results are reliable and cause lesser harm to the animal than the invasive telemetry measurement (Wilde et al., 2017).

Previous experimental studies have reported 1.3 to 2.8-fold rise in circulating UA levels in rats fed with 2% Oxo, which is well in agreement with the 2.4 to 3.4-fold increases in the current studies (Kang, 2002; Mazzali et al., 2002; Sanchez-Lozada et al., 2005). Notably, some of the UA variation between the present and previous studies is likely due to different administration routes of Oxo, as some studies have employed gastric gavage technique instead of oral administration (Sanchez-Lozada et al., 2005; Sánchez-Lozada et al., 2002). While with free accessible digestion the accurate intake of Oxo cannot be quite accurately assessed, the method is more physiological than invasive gastric gavage, and therefore its use is ethically more justifiable. Gastric gavage carries a risk for aspiration and organ injuries, and the associated increase in stress levels can influence the measured variables. As an example, pituitary release of adrenocorticotropic hormone in response to stress stimulates adrenal gland to release glucocorticoids, most importantly cortisol, which can further elicit multiple systemic effects, such as mobilization of amino acids, which then can serve as precursor for UA (Brown et al., 2000). Treatment of hyperuricemia was not included in the study protocol, as previous studies have repeatedly shown the efficacy of XO-inhibitors and uricosuric agents in preventing the pathological changes associated with Oxo feeding (Mazzali et al., 2002; Mazzali, Hughes, et al., 2001; Sanchez-Lozada et al., 2005; Sánchez-Lozada, Soto, et al., 2008).

While the applied Oxo model is the oldest and probably also the most used rodent model of hyperuricemia, it can still be regarded as a valid experimental approach for investigating the physiological effects of hyperuricemia. Another novel experimental model that uses uricase gene knock out mice has also been employed (Wu et al., 1994). In this model, mice carrying a mutated gene have 10-fold increases in serum UA levels, with more than half of the mice dying before four weeks of age. This highlights how ill equipped the rodent kidney is to handle UA levels that more closely resemble the human situation. Thus, any rodent model of hyperuricemia faces serious limitations when extrapolating the results to humans.

6.1.2 Clinical hemodynamic study (study IV)

Hemodynamic recordings were conducted in supine position by utilizing ICG_{WB} ($CircMon^{R}$) and pulse wave analysis from the radial artery. Stroke volume and CO recorded with $CircMon^{R}$ correspond to values obtained by utilizing 3-dimensional echocardiography and the thermodilution and direct oxygen Fick methods (T. Kööbi, Kaukinen, Ahola, et al., 1997; T. Kööbi, Kaukinen, Turjanmaa, et al., 1997; Koskela et al., 2013), and the PWV values correlate well with values measured with the current "gold standard" tonometric method (T. Kööbi et al., 2003; Wilenius et al., 2016). Thus, recording of the PWV with ICG_{WB} can be regarded as a validated method to evaluate large arterial stiffness.

Radial applanation tonometry was employed to measure radial and central BP and central wave reflection. The method enables continuous detection of radial pulse wave within each heartbeat, an advantage in when comparing with the commonly used method of recording only 10-20 pulse waveforms (Hayward et al., 2002).

6.2 Results and findings

6.2.1 The effect of experimental hyperuricemia on the cardiovascular system and the kidney antioxidant capacity and oxidative stress

In studies I and II the aim was to investigate the effects of Oxo induced hyperuricemia on vascular function, morphology, and cardiac load, while study III focused on the renal effects of hyperuricemia in healthy and subtotally nephrectomized rats. We also evaluated oxidative stress markers *in vivo,* as the role of oxidative stress has repeatedly been implicated in the renal and vascular pathologies associated with UA (Khosla et al., 2005). The power of the results is strengthened by the knowledge that all the studies employed the same animals, therefore excluding the effect of methodological and animal strain differences between the experiments. Indeed, variations in genes regulating urate homeostasis in the kidney and in the liver may significantly influence on the development of renal abnormalities (Preitner et al., 2009).

Carotid and mesenteric arteries were chosen for the functional studies as reduced compliance of the large arteries is an independent predictor of cardiovascular mortality in CKD, whereas morphological vascular changes often manifest in the small resistance-size arteries (London et al., 1996). The rationale to investigate the functional responses in both carotid and mesenteric arteries is based on the knowledge that in the carotid artery (study I) the vasorelaxation in mainly mediated via endothelium-derived NO, while in the mesenteric artery (study II), the relaxation is predominantly mediated by endothelium-independent hyperpolarization of the smooth muscle (Arvola, 1999; P. Kööbi et al., 2003). Arterial contractions were examined in each vessel to reveal the possible differences in vasoconstrictor sensitivity that might interfere with the interpretation of the relaxation responses. Based on these results, the observed changes in the vasorelaxation responses were not explained by differences in vasoconstrictor sensitivities.

In the carotid arteries of the NX rats, hyperuricemia associated with improved vasorelaxation mediated via endothelial (Ach) and exogenous (SNP) NO, and plasma UA concentration correlated positively with maximal Ach-elicited vasorelaxation (study I). In all groups, the presence of the NOS inhibitor L-NAME practically abolished the relaxations to Ach, supporting the previous observations of NO being the most important vasodilatory agent in the rat carotid artery. In contrast to the carotid artery, in the main branch of the mesenteric artery (study II), endothelium-dependent vasorelaxation was not influenced by UA, whereas endothelium-independent vasodilatation induced by BK_{Ca} channel opener NS-1619 was impaired in the NX+Oxo rats. As in the carotid artery, the changes in vasorelaxation responses were observed only in uremic milieu, as no differences were observed in healthy hyperuricemic rats. Collectively, the results from the functional vascular studies suggest that, irrespective of the vessel studied, the effects of UA become more pronounced in states of increased oxidant burden, as in CKD.

The role of UA in histological renal scarring has been implicated in human and in experimental studies, while in the present low renin model of CKD, elevated circulating UA levels had beneficial effects on kidney morphology (study III) (Myllymäki et al., 2005). Generally, the mechanisms via which UA might exert detrimental renal effects, involve both crystal and non-crystal pathways, the latter consisting of hemodynamic (renovascular) and non-hemodynamic changes (inflammation, oxidative stress, impaired endothelial NO production and vasoconstriction). A recent study using a mouse model of aristolochic acid-induced

CKD concluded that urinary acidification that leads to hyperuricemia and UA crystalluria is a necessary step for the pathological actions of UA to manifest in the kidney (Sellmayr et al., 2020).

A likely explanation for the beneficial vascular and renal effects observed in the hyperuricemic NX rats may relate to increased antioxidant capacity and reduced oxidative stress *in vivo*, as evidenced by increased plasma TRAP, and reduced urinary excretions of 8-isoprostaglandin $F_{2\alpha}$ and 11-epi-prostaglandin $F_{2\alpha}$. Notably, 8-isoprostaglandin $F_{2\alpha}$ excretion was not increased during the three weeks' postoperative recovery period, while 11-epi-prostaglandin $F_{2\alpha}$ excretion was elevated already in the early course of CKD. Increased oxidative stress is not therefore necessarily a straightforward result of renal mass reduction but rather develops later in the disease. Increased 11-epi-prostaglandin $F_{2\alpha}$ excretion may also indicate renal mast cell activation or COX-2 induced prostanoid synthesis, not necessarily oxidative stress *per se* (Mucha & Riutta, 2001; Pugliese et al., 1985; Vainio et al., 2003).

Uric acid could reduce oxidative stress by scavenging various cell damaging ROS, such as superoxide, hydroxyl radical, and singlet oxygen. The superoxide radical is of particular interest, as it can react with NO and reduce its bioavailability in various essential cellular functions, including endothelium-mediated vasodilation. If not inhibited by UA, the reaction product of superoxide anion and NO generates the toxic peroxynitrite ($ONOO^-$), which can further form secondary toxic radicals (Squadrito et al., 2000). Uric acid can also maintain the activity of superoxide dismutase, an enzyme that dismutates superoxide into non-toxic oxygen and hydrogen peroxide (H_2O_2) (Hink H. Ulrich et al., 2002), and by such means prevent the harmful interaction of superoxide radical with NO. Thus, the beneficial antioxidant actions of UA are largely coupled to increased availability of NO and preserved endothelial function. As in the NO-mediated responses, effect of UA on endothelium-independent relaxations observed in the mesenteric artery might be coupled to reduced ROS concentration, as ROS are known to inhibit BK_{Ca} (Gutterman et al., 2005). Furthermore, increased bioavailability of H_2O_2 can modulate the open probability of the BK_{Ca} channels, as H_2O_2 can induce vasodilatation directly via BK_{Ca} activation, especially under conditions of reduced NO availability like in CKD (Gutterman et al., 2005; Wei et al., 1996).

6.2.2 The association of plasma UA levels with arterial stiffness

Despite several previous studies, the association between UA and arterial stiffness is still controversial. At least in part, this controversy likely reflects the differences in the methodology and definitions of hyperuricemia, as well as heterogeneity of the study populations: different ethnicities, cardiovascular risk profiles, medications, and lifestyle habits (Albu et al., 2020). Unlike in many previous studies, medicated hypertensive patients were excluded in the current study population. A direct association between plasma UA and PWV still remained, suggesting that in this normouricemic population, the association between UA and large arterial stiffness was not explained by the presence of hypertension, but was directly related to UA.

The mechanisms by which UA could cause arterial stiffening in humans might involve endothelial dysfunction, initially triggered by oxidative stress and endothelial NO depletion, similarly as in experimental animals. The association between UA and endothelial dysfunction has been evidenced with medicated hypertensive females in whom lower baseline circulating UA levels correlated with better endothelial function (Tanaka et al., 2018). Furthermore, allopurinol was found to improve endothelium-dependent vasodilatation in the forearm in subjects with chronic heart failure and mildly hypertensive type 2 diabetes patients (R. Butler et al., 2000; Farquharson et al., 2002). However, in some studies this association has been inverse. In patients with type I diabetes, and also in regular smokers, acute intravenous UA administration improved Ach-induced vasodilatation, while in genetically hypouricemic subjects impaired flow-mediated dilatation in the brachial artery was observed (Sugihara et al., 2015; Waring et al., 2006). Circulating UA might also induce atherosclerosis in the vasculature by directly stimulating macrophages causing inflammation, production of cytokines and collagen, and smooth muscle cell proliferation (Gicquel et al., 2015).

The effect of UA lowering therapy on arterial stiffness has also been explored in interventional studies. One meta-analysis found a favorable effect of allopurinol on AIx but not on aortic PWV. The authors discussed that the finding was related to decreased oxidative stress and improved endothelial function, with a predominant influence on wave reflection (Deng et al., 2016). There are also few studies in diabetic subjects reporting beneficial effects of selective sodium-glucose cotransporter-2 inhibitors on arterial stiffness. Aside from BP-lowering effect, lower levels of circulating UA due to secondary uricosuric effect of selective sodium-glucose

cotransporter-2 inhibitors was proposed as one possible mechanism for the reduced arterial stiffness (Chilton et al., 2015; Sanchez et al., 2018).

6.3 Is there a causal role of uric acid in renal disease?

While most epidemiological and experimental studies conducted so far rather unambiguously support the association between high UA levels and renal disease, the causality of this relationship is after years of intensive research still controversial. The causal nature of the evidence can be weighed against Bradford Hill's criteria (A. B. Hill, 1965).

Strength: The most comprehensive systematic meta-analyses concluded that convincing evidence over the role of UA in disease states only exists for gout and nephrolithiasis (X. Li et al., 2017).

Consistency: Studies exploring the association between UA and kidney disease have reported inconsistent results in different study populations. For instance, in healthy subjects, elevated UA level is generally coupled to renal disease risk (Hsu et al., 2009; L. Li et al., 2014; Weiner et al., 2008), but in subjects with pre-existing CKD the results have been controversial (Srivastava et al., 2018; Sturm et al., 2008). In ESRD patients, J-shaped mortality relationship has been observed, with both high and low levels of circulating UA levels conveying an increased cardiovascular risk (Suliman et al., 2006).

Specificity: Hyperuricemia is frequently associated with a range of cardiovascular co-morbidities and therefore, the effects of UA *per se* are difficult to point out. Enhanced UA generation via XO-pathway accompanied by cell-toxic ROS generation often coexists with reduced renal UA excretion, both contributing to elevation of circulating UA concentration.

Temporality: In healthy population higher UA levels have predicted future development of CKD, while in individuals with pre-existing CKD, high circulating UA level does not independently result in disease progression. Evaluation of temporal relationship between UA and renal manifestations is mixed by cofounding factors (such as lifestyle and dietary habits), which are difficult to standardize in long

clinical trials. Furthermore, the time for an end-organ damage to develop is often decades, making it harder to draw conclusions from the causal relationship.

Experimental evidence: A large body of experimental and biologically plausible evidence supports the causal role of UA in renal disease. It should be acknowledged, however, that the metabolism of UA in human is different from species used in experimental studies and the experimental evidence should be interpreted considering this biological fact.

6.4 Future perspectives for interventional studies

One way to assess the role of UA in diseases is to conduct an interventional study to explore whether lowering UA would improve the outcome of interest. So far, the two largest randomized, double-blinded, placebo-controlled studies of the matter did not report any benefit of allopurinol use in slowing down the progression of CKD. In fact, when the studies were combined, there was a tendency for higher mortality in the intervention groups (Badve et al., 2020; Doria et al., 2020). Both of these studies excluded patients with gout and therefore the results from these studies can only be applied to normouricemic CKD patients (Goldberg et al., 2021). The interpretation of the results is also conflicted by a wide use of RAAS blockers by the study participants. If, according to the current theory, UA would mediate its detrimental effects via RAAS activation, blockage of the RAAS-system would secondarily prevent the harmful effects of UA as well, masking the potential effects of XO-inhibitors. In the future, studying the effects of urate lowering therapy in hyperuricemic (either with or without symptoms) CKD patients are warranted.

It is plausible that the key factor in determining whether high UA levels oppose a risk for renal disease is the etiological background of hyperuricemia. As most of the harmful effects of UA are mediated via intracellular, not circulating UA, factors that increase intracellular UA generation, such as enhanced XO-activity for any reason or ingestion of fructose, would be expected to have negative health consequences. In contrast, passive UA retention solely due to impaired kidney function might not be as harmful and might even be beneficial. This could explain why hyperuricemia in the settings of normally functioning kidneys is more accurate in predicting the future development of CKD, while in subjects with pre-existing CKD UA level has less prognostic significance. Thus, among hyperuricemic subjects

two separate phenotypes are represented: the overproducers and the underexcretors. It should be emphasized, however, that these phenotypes overlap, and in most cases both increased production and underexcretion are equally present.

6.5 Concluding remarks

The human mind is keen to find simple solutions to complex problems. There is also a natural tendency for dualistic categorization of things to be either "good" or "bad" while in most cases, the truth likely lies somewhere in between these extremes. An idea of an easily accessible, inexpensive, and unambiguously interpretable risk marker, whether it be a single genetic variant or a metabolic substance like UA, is therefore attractive. Such a marker could ideally be utilized in clinical decision making when assessing the risk and prognosis of an individual patient, as well as in targeting the medical interventions more precisely. In living complex organisms, however, such diagnostic shortcuts are rare.

The spectrum of human diseases ranges from rare monogenic to common multifactorial diseases, such as CKD. In the latter end of the scale both genetic, environmental and lifestyle factors play a role in disease development. Chronic kidney disease is often the final endpoint that results from other conditions, most importantly type 2 diabetes or hypertension, both of these conditions also having many shared overlapping risk factors. In contrast to many monogenic diseases, in which a single gene test can tell a lot about a person's risks and prognosis, in multifactorial diseases such test is unlikely ever to be found. Instead, the risk assessment should be based on consideration of various factors that modulate the individual risk. Based on the current evidence, measurement of circulating UA in asymptomatic individuals with pre-existing or high risk for cardiovascular or renal disease can be used in overall risk evaluation, and perhaps in the case of hyperuricemia, as a tool to motivate lifestyle change. In asymptomatic subjects, however, high UA levels should be interpreted cautiously with the focus on other more severe risk factors. Preventing and managing these co-morbidities should be the primary goal in the treatment of hyperuricemic subjects, while pharmacological treatment should generally be limited only to patients with symptomatic hyperuricemia (gout or UA nephrolithiasis).

While some authorities in the field of UA research have labeled UA as a detrimental substance, others justifiably question this view. In many fields of sciences, there is a risk that evidence contradicting with the generally accepted view will be ignored resulting in selective publication of studies, creating a self-perpetuating publication bias. This phenomenon is called "premature closure", a rejection of new information after a plausible hypothesis has been build. This is one of the many heuristic shortcuts in human decision making, extensively discussed in a widely acclaimed book "Thinking fast and slow" written by the Nobel winning author Daniel Kahneman. Other cognitive biases discussed in the book include availability heuristic, referring to the tendency to weigh the likelihood of things by the ease they are recalled from the memory, a phenomenon reinforced by the publication bias. The overestimation of evidence that supports the initial hypothesis and underestimation of the evidence against it is referred as anchoring effect. Pre-existing beliefs influence new data procession, making data supporting the pre-existing conceptions more easily adoptable than data that contradicts with them, and this constitutes the confirmation bias. By approaching scientific questions holistically will minimize the risk of bias and most likely lead to the most valid conclusion.

7 SUMMARY

The present series of studies consists of experimental (studies I, II and III) and clinical part (study IV). The aim of the experimental studies was to examine the cardiovascular and renal effects of 9 weeks dietary 2% oxonic acid-induced hyperuricemia in the 5/6 NX model of CKD. In the clinical study, the association of plasma UA level with various hemodynamic variables was evaluated in normotensive or never-medicated hypertensive volunteers.

The main findings and conclusions of the present study are:

1. Surgically nephrectomized rats demonstrated characteristics of moderate renal impairment: reduced endothelium-dependent vasorelaxation responses in the carotid and mesenteric arteries *in vitro*, hypertrophic remodeling of the resistance size mesenteric arteries, mild hypertension, cardiac volume load, histological renal damage, and increased systemic and renal oxidative stress.

2. Experimental hyperuricemia induced by 2% oxonic acid feeding was characterized by reduced oxidative stress and increased antioxidant capacity *in vivo*, as indicated by reduced 24-hour urinary excretion of 11-epi-prostaglandin $F_{2\alpha}$ and 8-isoprostaglandin $F_{2\alpha}$, and increased plasma TRAP, respectively.

3. In CKD, NO-mediated carotid artery relaxation was improved, and endothelium-independent mesenteric artery vasodilatation was impaired during hyperuricemia, but no differences in the arterial vasorelaxation were observed in rats with preserved renal function. Maximal wall tension or sensitivity of the constrictor responses in conduit size or resistance size mesenteric arteries was not influenced by UA. The effects of hyperuricemic milieu on vascular function may depend on the vascular bed studied and the effects are also influenced by the uremic milieu.

4. The kidneys of the hyperuricemic rats demonstrated improved glomerular and tubular histology, reduced renal fibrosis, and inflammation. The favorable renal effects of hyperuricemia were associated with reduced intrarenal oxidative stress and were specifically related to CKD.

5. Hyperuricemia did not influence cardiac volume load as indicated by ventricle weights and ventricular ANP, BNP, SkαA, and β-MHC mRNA expressions.

6. In normotensive and never-treated hypertensive volunteers, higher plasma UA levels were associated with increased large arterial stiffness, as indicated by increased PWV. Plasma UA was an independent explanatory factor for aortic to popliteal PWV, but not for other hemodynamic variables or BP. In humans, UA might predispose to cardiovascular disease by increasing large arterial stiffness, even at plasma UA levels predominantly within the normal reference range.

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9 ORIGINAL PUBLICATIONS

PUBLICATION I

Hyperuricemia, oxidative stress, and carotid artery tone in experimental renal insufficiency

Venla Kurra, Arttu Eräranta, Pasi Jolma, Tuija I. Vehmas, Asko Riutta, Eeva Moilanen, Anna Tahvanainen, Jarkko Kalliovalkama, Onni Niemelä, Juhani Myllymäki, Jukka Mustonen and Ilkka Pörsti

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Hyperuricemia, Oxidative Stress, and Carotid Artery Tone in Experimental Renal Insufficiency

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BACKGROUND

Hyperuricemia may play a role in the pathogenesis of cardiovascular disease, but uric acid is also a significant antioxidant. We investigated the effects of oxonic acid–induced hyperuricemia on carotid artery tone in experimental renal insufficiency.

METHODS

Three weeks after 5/6 nephrectomy (NX) or Sham operation, male Sprague-Dawley rats were allocated to 2.0% oxonic acid or control diet for 9 weeks. Blood pressure was monitored using tail-cuff, isolated arterial rings were examined using myographs, and blood and urine samples were taken, as appropriate. Oxidative stress and antioxidant status were evaluated by measuring urinary 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) excretion and plasma total peroxyl radical-trapping capacity (TRAP), respectively.

RESULTS

Plasma creatinine was elevated twofold in NX rats, but neither NX nor oxonic acid diet influenced blood pressure. Urinary 8-iso-$PGF_{2\alpha}$ excretion was increased over 2.5-fold in NX rats on control diet. Oxonic acid diet increased plasma uric acid 2–3-fold, TRAP 1.5-fold, and reduced urinary 8-iso-$PGF_{2\alpha}$ excretion by 60–90%. Carotid vasorelaxation to acetylcholine *in vitro*, which could be abolished by nitric oxide (NO) synthase inhibition, was reduced following NX, whereas maximal response to acetylcholine was augmented in hyperuricemic NX rats. Vasorelaxation to nitroprusside was impaired in NX rats, whereas oxonic acid diet increased sensitivity also to nitroprusside in NX rats.

CONCLUSIONS

Oxonic acid–induced hyperuricemia reduced oxidative stress *in vivo*, as evaluated using urinary 8-iso-$PGF_{2\alpha}$ excretion, increased plasma TRAP, and improved NO-mediated vasorelaxation in the carotid artery in experimental renal insufficiency.

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Hyperuricemia is a typical finding in subjects with metabolic syndrome and renal disease.[1,2] Elevated uric acid correlates with increased cardiovascular mortality,[3] but the contribution of uric acid to the pathogenesis of cardiovascular disease remains controversial.[1] However, hyperuricemia may predispose to hypertension by increasing renal sodium reabsorption.[4,5]

Experimental studies, where rats were made hyperuricemic by the uricase inhibitor oxonic acid, have suggested that high uric acid may play a causal role in the development cardiovascular disease.[6–9] Oxonic acid diet has resulted in renal injury consisting of afferent arteriolopathy and renal cortical vasoconstriction, glomerulosclerosis, and increased fibrosis.[6,8,9] The hyperuricemia-induced renal vasculopathy could be prevented by blockade of the renin–angiotensin system, stimulation of nitric oxide (NO) synthesis with dietary L-arginine, or administration of the superoxide scavenger, tempol.[7,10,11] Increased uric acid concentration has also been suggested to impair NO production in cultured endothelial cells *in vitro*, and hyperuricemic rats have shown decreased NO concentration in serum.[12] Collectively these findings suggest that the detrimental effects of uric acid may be mediated via impaired NO-mediated responses or enhanced renin–angiotensin system activity.

High uric acid concentration is a risk factor for atherosclerosis,[13] whereas hyperuricemia is a predictor of stroke in patients with type 2 diabetes.[14] Increased amounts of uric acid have been found in atherosclerotic plaques, suggesting a role for uric acid in the etiology of atherosclerosis.[15] Yet, uric acid is an antioxidant, as its soluble form urate can scavenge various radicals.[16,17] Uric acid provides protection against oxidative stress induced by peroxynitrite, a toxic product formed in the reaction between superoxide anion and NO.[17] Uric acid also prevents the degradation of extracellular superoxide dismutase, an enzyme maintaining normal NO levels and endothelial function.[18] The antioxidant properties of uric acid are in conflict with the concept that hyperuricemia *per se* induces vascular damage.

Despite the several vascular associations and actions of uric acid, the effects of hyperuricemia on arterial tone have only

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been examined in the glomerular afferent arteriole.[8] Here, we tested the hypothesis whether oxonic acid–induced hyperuricemia influences arterial tone in experimental chronic renal insufficiency (CRI), and subjected 5/6 nephrectomized and Sham-operated rats to 2.0% oxonic acid diet. This model of CRI is characterized by increased oxidative stress,[19] impaired endothelial function, but only a modest increase in blood pressure 12 weeks after subtotal nephrectomy.[20–22] The present results show that carotid artery relaxation via NO was impaired in CRI, whereas oxonic acid diet improved vasorelaxation, possibly because of the antioxidant properties of uric acid.

METHODS

Animals and experimental design. Male Sprague-Dawley rats were subjected to 5/6 nephrectomy (NX) or Sham-operation at the age of 8 weeks under ketamine/diazepam anesthesia (75 and 2.5 mg/kg intraperitoneally, respectively). In NX rats, the upper and lower poles of the left kidney were cut off, and the right kidney was removed.[20,21] In the Sham groups, both kidneys were decapsulated. Antibiotics (metronidazole 60 and cefuroxime 225 mg/kg) were given postoperatively, and pain was relieved with buprenorphine (0.2 mg/kg subcutaneously, three times a day, 3 days). Three weeks later, the rats were divided into four groups (n = 10 in each) so that mean systolic blood pressures, body weights, and urine volumes were similar in the two Sham (Sham, Sham+Oxo) and the two NX groups (NX, NX+Oxo), respectively. Systolic blood pressures were measured at +28 °C by the tail-cuff method as averages of five recordings in each rat (model 129 blood pressure meter; IITC, Woodland Hills, CA) (flowchart in **Figure 1**).

All groups were given standard chow (lactamin R34; AnalyCen, Lindköping, Sweden). After the 3rd study week, oxonic acid (20 g/kg chow; Sigma-Aldrich Chemical, St Louis, MO) was supplemented in the food of the Sham+Oxo and NX+Oxo groups. Hyperuricemia was confirmed by tail vein samples at study week 5, and 24-h urine was collected in metabolic cages at the end of the 2nd and 11th study weeks (**Figure 1**).

After 9 diet weeks, the rats were weighed and anesthetized (urethane 1.3 g/kg), and blood samples from carotid artery for plasma creatinine, urea, uric acid, lipids, and total peroxyl radical-trapping capacity (TRAP) measurements were drawn into chilled tubes with heparin or EDTA as anticoagulants. The heart and kidneys were removed and weighed, and the kidneys were fixed in 4% formaldehyde for 24 h, and embedded in paraffin. The experiments were approved by the Animal Experimentation Committee of the University of Tampere, Finland, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Carotid artery responses in vitro. The carotid artery was chosen for the studies because of the controversial results concerning uric acid, central circulation, and stroke.[14,17,23–25] In the rat carotid artery, the vasorelaxation to acetylcholine (Ach) is largely mediated via endothelium-derived NO,[26] which probably makes this vessel sensitive to changes in the antioxidant status *in vivo*.[19] A 2-mm-long standard section of left carotid artery from each animal was prepared, and suspended between hooks in an organ bath chamber in physiological salt solution (pH 7.4) containing (mmol/l): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, and $MgSO_4$ 1.2. The physiological salt solution was aerated with 95% O_2 and 5% CO_2 and the ring was equilibrated for 1½ h at +37 °C with a resting preload of 3.7 mN/mm to induce maximal contractile force generation in the carotid ring,[26] measured using isometric force–displacement transducers (FT 03, 7 E Polygraph; Grass Instrument, Quincy, MA).

The rings were allowed 30 min at baseline tension in between each concentration–response challenge. Contractions to noradrenaline (NA) were cumulatively elicited, and relaxations to the NO donor nitroprusside and β-adrenoceptor agonist isoprenaline were examined in rings precontracted with 1 μmol/l NA. The relaxations to Ach, in the absence and presence of the NO synthase inhibitor N^G-nitro-L-arginine

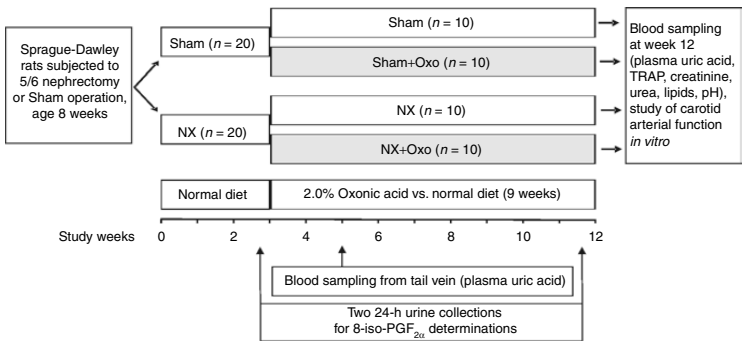

Figure 1 | Flowchart of the study. 8-iso-$PGF_{2\alpha}$, 8-isoprostaglandin $F_{2\alpha}$; NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat; TRAP, total peroxyl radical-trapping capacity.

methyl ester (L-NAME, 0.1 mmol/l), were examined in rings precontracted with 1 μmol/l NA.

Plasma creatinine, urea, uric acid, TRAP, urine 8-isoprostaglandin $F_2\alpha$ excretion, and glomerular histology. Plasma and urine creatinine was determined using Jaffe's colorimetric assay, and plasma urea using colorimetric enzymatic dry chemistry (Vitros 950 analyzer; Johnson & Johnson Clinical Diagnostics, Rochester, NY). Uric acid was measured by an enzymatic colorimetric method,[27] and triglycerides, total and high-density lipoprotein (HDL) cholesterol concentrations were analyzed using Cobas Integra 800 automatic analyzer (Hoffman-La Roche, Basel, Switzerland). Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol.

Plasma TRAP was measured using luminol-enhanced chemiluminescence, based on peroxyl radical production by decomposition of 2,2-azo-bis(2-aminopropane) hydrochloride (Polysciences, Warrington, PA), as previously described.[28,29] The 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) assay was also performed as previously described.[30] Briefly, urine was extracted on a C_2 silica cartridge (Applied Separation, Allentown, PA) and 8-iso-$PGF_{2\alpha}$ was determined using 8-iso[^{125}I]-$PGF_{2\alpha}$ radioimmunoassay.

Five-micrometer-thick kidney sections were stained with hematoxylin–eosin and processed for light microscopic evaluation by an expert blinded to the treatments. The glomerulosclerosis score for each animal was derived as the mean of all sample glomeruli: 0 = normal, 1 = mesangial expansion or basement membrane thickening, 2 = segmental sclerosis <25% of the tuft, 3 = segmental sclerosis 25–50% of the tuft, 4 = diffuse sclerosis >50% of the tuft, 5 = diffuse glomerulosclerosis, total tuft obliteration and collapse.[22]

Data presentation and analysis of results. The contractile responses were expressed as wall tension (mN/mm) and as percentage of maximum. The vasorelaxations were presented as a percentage of preexisting contractile force. Statistical analyses were by one-way and two-way analyses of variance (ANOVAs), as appropriate, and the least significant difference test was used for post hoc analyses (SPSS 11.5; SPSS, Chicago, IL). ANOVA for repeated measurements was applied for data consisting of repeated observations at successive observation points. Spearman's two-tailed correlation coefficient (r) was used, and the results were expressed as means and s.e.m. $P < 0.05$ was considered significant. Unless otherwise indicated the P values in the text refer to one-way ANOVA.

Drugs. Ketamine (Parke-Davis Scandinavia, Solna, Sweden), cefuroxime, diazepam (Orion Pharma, Espoo, Finland), metronidazole (B. Braun, Melsungen, Germany), buprenorphine (Reckitt & Colman, Hull, England), acetylcholine chloride, isoprenaline hydrochloride, N^G-nitro-L-arginine methyl ester, NA bitartrate (Sigma-Aldrich Chemical, St Louis, MO), and sodium nitroprusside (Fluka Chemie, Buchs, Switzerland) were used.

RESULTS

Blood pressure, heart and body weights, and glomerulosclerosis

Systolic blood pressure was not significantly influenced by the oxonic acid diet or NX (**Table 1**). However, heart weight to body weight ratio was higher in the NX and NX+Oxo groups when compared with Sham rats ($P = 0.004$ and 0.001, respectively). Average body weight gain was comparable in all groups (**Table 1**). Histological analysis revealed that the glomerular damage index was lower in the NX+Oxo than the NX group (1.1 ± 0.4 vs. 1.8 ± 0.2, $P < 0.05$), whereas the Sham and Sham+Oxo groups were completely devoid of glomerular changes (damage index was 0 in both groups).

Laboratory findings

At the end of the study, the oxonic acid diet elevated plasma uric acid concentration 3.4-fold in the Sham+Oxo rats and 2.4-fold in the NX+Oxo rats, respectively (**Table 2**). Plasma creatinine and urea concentrations were approximately two-fold higher in the NX rats when compared with Sham, and were not affected by oxonic acid diet. Creatinine clearance was reduced by ~60% in both NX groups, and also by 33% in hyperuricemic Sham rats.

Before the oxonic acid diet was started, no significant differences were found in the 24-h urinary excretion of 8-iso-$PGF_{2\alpha}$, a marker of oxidative stress *in vivo* (**Figures 1** and **2a**).[31] During the 9th week on the 2.0% oxonic acid diet, the 24-h excretion of 8-iso-$PGF_{2\alpha}$ was increased in the NX group, but was reduced by ~60 and 90% in the Sham+Oxo and NX+Oxo groups, respectively, when compared with their respective controls (**Figures 1** and **2b**).

At the end of the study, the plasma concentrations of TRAP were increased 1.5-fold in groups ingesting the oxonic acid diet (**Table 2**), and there was a linear correlation between plasma uric acid concentration and TRAP in all groups (**Figure 2c**). Plasma total cholesterol, HDL cholesterol, and non-HDL cholesterol concentrations were elevated in NX rats (**Table 2**). When analyzed using two-way ANOVA, an elevation of plasma triglycerides was also uncovered in NX rats when compared

Table 1 | Experimental group gross data (oxonic acid feeding period from week 3 to 12)

	Sham	Sham+Oxo	NX	NX+Oxo
Systolic blood pressure (mm Hg)				
Week 3	122 ± 4	122 ± 5	127 ± 5	127 ± 7
Week 12	139 ± 7	137 ± 6	140 ± 7	149 ± 5
Heart/body weight (g/kg)	3.99 ± 0.05	4.17 ± 0.10	5.09 ± 0.30*	5.25 ± 0.39*
Body weight (g)				
Week 3	333 ± 5	334 ± 6	327 ± 8	330 ± 7
Week 12	416 ± 8	404 ± 9	430 ± 11	409 ± 6

Values are mean ± s.e.m., $n = 10$ for all groups.
NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat.
*$P < 0.05$ compared with the Sham group.

Table 2 | Plasma uric acid after 2 and 9 weeks of the oxonic acid diet (study weeks 5 and 12), and plasma chemistry and blood pH after the 12-week study in the experimental groups

	Sham	Sham+Oxo	NX	NX+Oxo
Uric acid (μmol/l)				
Week 5	50.3 ± 13.0	106.8 ± 15.5*	49.1 ± 10.2	86.6 ± 9.6*
Week 12	37.4 ± 13.4	126.4 ± 24.2*	65.9 ± 23.2	156.6 ± 22.7*,**
Creatinine (μmol/l)	37.8 ± 5.8	48.8 ± 3.6	81.6 ± 3.9*	84.1 ± 9.8*
Creatinine clearance (ml/min)	3.0 ± 0.4	2.0 ± 0.2*	1.2 ± 0.1*	1.2 ± 0.1*
Urea (mmol/l)	6.8 ± 0.4	8.6 ± 0.5	13.7 ± 1.0*	15.1 ± 2.4*
TRAP (μmol/l)	422 ± 52	611 ± 70*	491 ± 70	714 ± 60*,**
Cholesterol (mmol/l)	2.25 ± 0.10	2.14 ± 0.13	4.80 ± 0.47*	5.09 ± 0.65*
HDL (mmol/l)	1.64 ± 0.09	1.62 ± 0.10	3.55 ± 0.37*	3.50 ± 0.41*
Non-HDL (mmol/l)	0.61 ± 0.03	0.51 ± 0.04	1.25 ± 0.21*	1.59 ± 0.27*
Triglycerides (mmol/l)	1.16 ± 0.11	0.96 ± 0.08	1.46 ± 0.09***	1.77 ± 0.34***
Blood pH	7.44 ± 0.04	7.39 ± 0.03	7.37 ± 0.04	7.41 ± 0.03

Values are mean ± s.e.m., $n = 10$ for all groups.
HDL, high-density lipoprotein; NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat; TRAP, total peroxyl radical-trapping antioxidant capacity.
*$P < 0.05$ compared with the Sham group, **$P < 0.05$ compared with the NX group, ***$P < 0.05$ NX groups compared with the Sham groups using two-way analysis of variance.

with Sham rats ($P = 0.006$, two-way ANOVA). No differences in blood pH were detected (**Table 2**).

Functional responses of isolated carotid arterial rings

Contractile responses to NA were similar between the study groups (**Figure 3a,b**). Thus, differences in vasoconstrictor sensitivity cannot explain the differences in vasorelaxations.

The relaxations to Ach were reduced in NX rats when compared with Sham rats, whereas the maximal response to Ach was improved in the NX+Oxo group when compared with the NX group ($P = 0.027$; **Figure 4a**). Hyperuricemia had no effect on the response to Ach in Sham rats. In both NX and NX+Oxo groups, plasma uric acid concentration correlated with the maximal relaxation to Ach (**Figure 2d**), whereas no correlation was observed between maximal Ach response and plasma levels of creatinine, urea, lipids, or blood pressure (data not shown). Plasma TRAP also correlated with maximal Ach response in NX rats ($r = 0.73$, $P = 0.026$).

The NO synthase inhibitor L-NAME abolished the relaxation to Ach in all groups (**Figure 4b**), indicating that the response was mediated via NO. The relaxations to nitroprusside were also reduced in both NX groups vs. Sham groups, whereas the reduction was less marked in the NX+Oxo group (**Figure 4c**). Higher relaxation to 10 nmol/l nitroprusside was detected in the NX+Oxo group when compared with the NX group ($P = 0.027$). Vasorelaxation induced by isoprenaline was similar in all groups (**Figure 4d**).

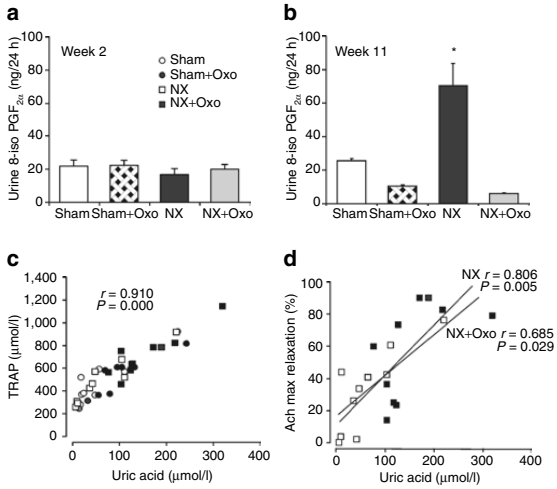

Figure 2 | Bar graphs show 24-h urinary excretion of 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) (**a**) before the 2.0% oxonic acid feeding period at the end of study week 2, and (**b**) during the 2.0% oxonic acid diet at the end of study week 11. Scatter plots show (**c**) plasma uric acid and total peroxyl radical-trapping capacity (TRAP), and (**d**) plasma uric acid and maximal relaxation induced by Ach after 12 study weeks in the experimental groups; *$P < 0.05$ vs. the Sham group, †$P < 0.05$ vs. the NX group. NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat.

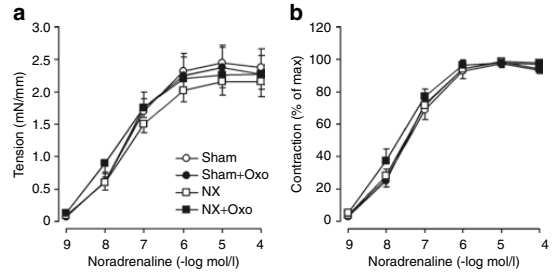

Figure 3 | Line graphs show contractile responses induced by noradrenaline as (**a**) developed tension and (**b**) percent of maximum in the experimental groups. NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat.

DISCUSSION

Here, we demonstrated for the first time that oxonic acid–induced hyperuricemia reduced oxidative stress and increased TRAP *in vivo* in the rat, as evaluated by urinary 8-iso-$PGF_{2\alpha}$ excretion and plasma TRAP, respectively. Higher uric acid concentration paralleled with improved carotid artery vasorelaxation via NO in NX rats, the improvement of which correlated significantly with plasma concentration of uric acid. These findings suggest that by counterbalancing the oxidative stress associated with CRI,[19] uric acid improved NO-mediated vasorelaxation independently of the level of blood pressure.

The oxonic acid diet induces hyperuricemia by inhibiting the hepatic uricase.[6–9] Previous studies with 2.0% oxonic acid in rats have demonstrated a 1.3- to 2.8-fold rise in serum uric

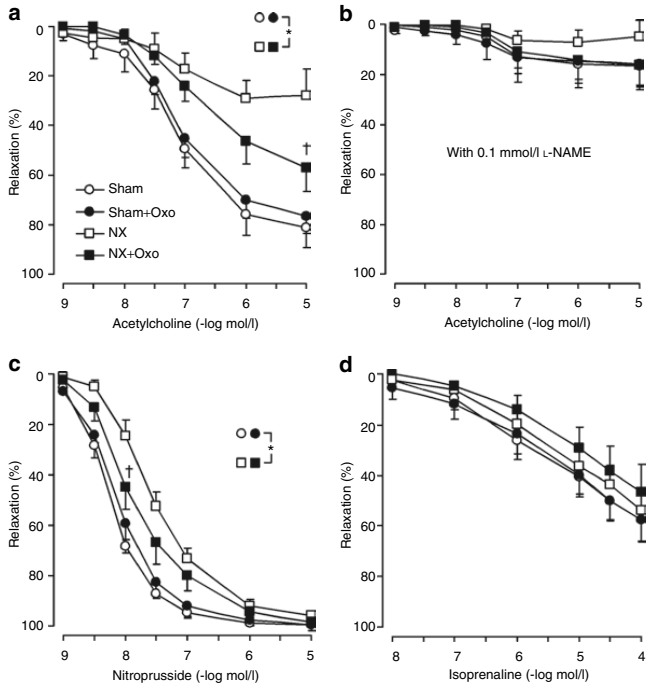

Figure 4 | Line graphs show relaxations induced by acetylcholine in the (**a**) absence and (**b**) presence of 0.1 mmol/l L-NAME, and relaxations elicited by (**c**) nitroprusside and (**d**) isoprenaline in the experimental groups; *$P < 0.05$, analysis of variance for repeated measurements; †$P < 0.05$ compared with the corresponding individual concentration in the NX group. NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, Sham-operated rat.

acid,[7–9] corresponding to the present 2.4- to 3.4-fold increase. Because allopurinol and uricosuric agents have been shown to prevent the pathological and pathophysiological changes induced by 2.0% oxonic acid feeding in several studies, treatment of hyperuricemia was not included in the present study.[6–9] The NX rats showed several characteristic findings of moderate renal insufficiency,[5,21,22] whereas the observed increase in heart weight is in agreement with the view that this model is characterized by volume overload.[21]

As our principal focus was on putative changes in NO-mediated vasorelaxation, we studied the functional responses in the carotid artery. In many vessels, the vasorelaxation to Ach is mediated via NO, prostacyclin, and endothelium-derived hyperpolarization,[32] but in the rat carotid artery the vasorelaxation is largely mediated via endothelium-derived NO.[26] We found that carotid vasorelaxation via both endothelial NO (Ach) and exogenous NO (nitroprusside) was impaired in CRI. Unexpectedly, oxonic acid diet improved vasorelaxation in response to NO in NX rats, and the plasma concentration of uric acid correlated positively with maximal Ach-mediated relaxation. In contrast, the vasorelaxation via cyclic AMP (isoprenaline) was not affected by oxonic acid feeding. Thus, the differences in carotid artery vasorelaxation were related to alterations in NO-mediated responses.

The present results appear to contradict with previous findings *in vitro* suggesting that uric acid inhibits NO production in cultured aortic endothelial cells,[12] and that uric acid induces oxidative stress through stimulation of renin–angiotensin system in cultured vascular smooth muscle cells.[33] In addition, experimental studies have suggested that uric acid is a mediator of cardiovascular and renal disease.[6–9,12] We recently found that experimental hyperuricemia elevated plasma aldosterone with a deleterious influence on sodium:potassium balance in rats with CRI. In that study, the Sham+Oxo rats also showed reduced creatinine clearance when compared with Sham rats, but no such reduction was observed in the NX+Oxo rats when compared with the NX group.[5] A similar finding was observed in this study, suggesting that this form of hyperuricemia is not associated with reduced glomerular filtration rate in NX rats. Altogether, the antioxidant properties of urate may mitigate the harmful influences of renin–angiotensin system stimulation associated with increased uric acid concentration. In addition, some of the differences between the results of the studies on oxonic acid–induced hyperuricemia may be related to the fact that 2.0% oxonic acid diet was used in some,[5–7,9] whereas administration of oxonic acid by gastric gavage (750 mg/kg/day) was used in others.[8,10,11] It seems likely that the effects of oxonic acid administration by gavage on the kinetics of plasma uric acid differ from those following 2.0% oxonic acid dietary intake.

Although hyperuricemia has in general been considered a cardiovascular risk factor,[1,15] conflicting data also exist. Uric acid serves as a scavenger of various free radicals.[16,17,34]

Intravenous uric acid infusion increases serum free-radical scavenging capacity in healthy volunteers,[35] and improves endothelial function in the forearm vascular bed of smokers and patients with type 1 diabetes.[36] Higher uric acid concentration is also associated with increased serum antioxidant capacity and reduced oxidative stress during acute physical exercise.[37] However, the antioxidant role of uric acid is complicated by the generation of superoxide anion as a by-product in the reaction of xanthine with xanthine oxidase to produce uric acid.[1] Furthermore, the reaction of superoxide with NO leads to the formation of the highly toxic peroxynitrite, with possible further formation of secondary free radicals.[17] Yet, uric acid can indirectly protect against the harmful effects of peroxynitrite by scavenging these secondary radicals.[17]

Increased oxidative stress is a characteristic feature of CRI.[19] Indeed, we observed a marked increase in the urinary 8-iso-$PGF_{2\alpha}$ excretion after 11 weeks of renal insufficiency in the present study, a finding that has not been reported previously in NX rats. Interestingly, 8-iso-$PGF_{2\alpha}$ excretion was not increased during the 3rd week after subtotal nephrectomy, indicating that increased oxidative stress is not a straightforward result of renal mass reduction but develops later in the course of chronically impaired renal function. It is of note that the beneficial influences were not limited to carotid vasorelaxation, as glomerulosclerosis was also alleviated by the oxonic acid–induced hyperuricemia in NX rats.

The levels of uric acid are higher in humans than in most other mammals.[1] Moreover, the relation of the uric acid levels with cardiovascular disease in humans has been suggested to show a J-shaped curve with the nadir of risk being in the second quartile.[1,3,38] The increased risk for the lowest quartile may reflect the decreased plasma antioxidant activity, whereas the increased risk at the higher levels may reflect the role of uric acid in inducing cardiovascular disease, leaving the antioxidant role of uric acid insignificant. In the 2.0% oxonic acid rat model of hyperuricemia, the levels of plasma uric acid are still rather low when compared with humans, and higher levels may be required to induce vascular damage in rats.

CRI is also associated with unfavorable changes in plasma lipids,[39] and impaired vasorelaxation could result from the increased level of low-density lipoprotein cholesterol, which inhibits endothelium-dependent vasodilatation.[40] The underlying mechanisms are impaired vascular NO production and increased superoxide anion generation that inactivates NO.[19] In line with these findings, plasma lipid values were increased by two- to threefold in the NX rats in the present study. Higher uric acid level may also provide antioxidant protection against the detrimental effects of low-density lipoprotein cholesterol on NO-mediated vasorelaxation.

In conclusion, we found that 2.0% oxonic acid diet which increased plasma uric acid, also reduced oxidative stress and increased peroxyl radical-trapping capacity *in vivo*, as evidenced by reduced 24-h urinary 8-iso-$PGF_{2\alpha}$ excretion and increased plasma TRAP, respectively. In parallel, oxonic acid diet enhanced vasorelaxation via NO in the carotid artery in experimental CRI, independently of the level of blood pressure and renal function. Therefore, this form of experimental hyperuricemia is not always associated with a deleterious influence on the cardiovascular system.

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PUBLICATION II

Effects of oxonic acid-induced hyperuricemia on mesenteric artery tone and cardiac load in experimental renal insufficiency

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Effects of oxonic acid-induced hyperuricemia on mesenteric artery tone and cardiac load in experimental renal insufficiency

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Abstract

Background: Recent studies suggest a causal role for increased plasma uric acid in the progression of chronic renal insufficiency (CRI). However, uric acid also functions as an antioxidant with possible beneficial effects.

Methods: We investigated the influence of hyperuricemia on mesenteric arterial tone (main and second order branch) and morphology in experimental CRI. Forty-four Sprague–Dawley rats were 5/6 nephrectomized (NX) or Sham-operated and fed 2.0% oxonic acid or control diet for 9 weeks.

Results: Oxonic acid feeding elevated plasma uric acid levels 2.4 and 3.6-fold in the NX and Sham groups, respectively. Plasma creatinine and urea were elevated 2-fold and blood pressure increased by 10 mmHg in NX rats, while hyperuricemia did not significantly influence these variables. Right and left ventricular weight, and atrial and B-type natriuretic peptide mRNA content were increased in NX rats, but were not affected by hyperuricemia. In the mesenteric artery, hyperuricemia did not influence vasoconstrictor responses *in vitro* to norepinephrine or potassium chloride. The small arteries of NX rats featured hypertrophic remodeling independent of uric acid levels: wall to lumen ratio, wall thickness and cross-sectional area were increased without changes in lumen diameter. In the main branch, vasorelaxations to acetylcholine were impaired in NX rats, but were not affected by hyperuricemia. In contrast, relaxations to the large-conductance Ca^{2+}-activated K^+-channel (BK_{Ca}) opener NS-1619 were reduced by oxonic acid feeding, whereas responses to nitroprusside were not affected.

Conclusions: Experimental hyperuricemia did not influence cardiac load or vascular remodeling, but impaired BK_{Ca} -mediated vasorelaxation in experimental CRI.

Keywords: Uric acid, Artery tone, Experimental chronic renal insufficiency, 5/6 nephrectomy, Oxonic acid

Background

The prevalence of increased plasma uric acid (UA), hyperuricemia, is high in patients with chronic renal insufficiency (CRI). The detrimental effects of hyperuricemia have been linked to cardiovascular complications, as high plasma UA levels commonly predict the development of hypertension [1] and the loss of renal function [2]. To date, however, the contribution of UA to cardiovascular disease has still remained controversial [3].

Previous experimental studies, carried out in rats made hyperuricemic by the inhibition of the UA degrading enzyme uricase using dietary 2.0% oxonic acid, have suggested a causal relationship between high UA and cardiovascular disease [4-7]. UA has been associated with stimulation of the renal renin-angiotensin system (RAS) and reduced nitric oxide (NO) synthesis. These mechanisms may have participated in the subsequent hypertrophic remodeling of the preglomerular arteries, tubulointerstitial damage, and thus predisposed to enhanced sodium retention [4-6,8]. Previously, incubation of the rat aortic rings with UA was found to reduce vasodilatation in response to acetylcholine (Ach) [9], while the endothelial NO production *in vitro* was

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reduced in hyperuricemic rats [10]. This suggests that the detrimental effects of hyperuricemia may partly result from endothelial dysfunction.

There is an ongoing debate about the role of UA in vascular disease. This arises from the ability of UA to reduce oxidative stress by preventing the superoxide radical from reacting with NO to generate peroxynitrite [11]. Peroxynitrite can impair NO-mediated relaxation by inhibiting a critical cofactor of the endothelial NO synthase [12], while it also can modulate vascular tone via smooth muscle [13]. In the aortas of apolipoprotein E-deficient mice, the ability of UA to reduce peroxynitrite levels was associated with improved Ach-elicited relaxation [12]. This result is in agreement with our earlier report that oxonic acid-induced hyperuricemia improved NO-mediated relaxation of the carotid artery in experimental CRI by alleviating oxidative stress [14].

In experimental CRI, reduced vasodilatation via Ca^{2+}-activated K^+-channels (BK_{Ca}), observed in isolated mesenteric arterial branches, may precede the elevation of blood pressure (BP) [15,16]. However, until now the vascular effects of UA have only been studied in arteries in which the endothelium-mediated dilatation is mainly mediated via NO [12,14]. Here we examined the tone of mesenteric arteries *in vitro* from the 5/6 nephrectomized (NX) and Sham-operated rats allocated to 2.0% oxonic acid diet for 9 weeks. Our findings suggest that oxonic acid diet impaired relaxation via BK_{Ca} in arterial smooth muscle, but did not significantly affect the endothelium-dependent responses, resistance artery structure, or cardiac load in experimental CRI.

Methods

Animals and experimental design

Male Sprague–Dawley rats (weight 335–341 g) were housed in standard animal laboratory conditions with free access to water and food pellets (Lactamin R34, AnalyCen, Lindköping, Sweden) containing 0.9% calcium, 0.8% phosphorus, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 1500 IU/kg vitamin D and 12550 kJ/kg energy, 16.5% protein, 4.0% fat, 58% nitrogen-free extract, 3.5% fiber, 6.0% ash, and 10% water. At the age of 8 weeks (study week 0), the rats were anesthetized with ketamine/diazepam (75 and 2.5 mg/kg, intraperitoneally, respectively) and NX (n = 22) was carried out by the removal of upper and lower poles of the left kidney and the whole right kidney. The sham-operation (n = 22) was performed by kidney decapsulation as previously described [15,17]. Antibiotics (metronidazole 60 mg/kg and cefuroxim 225 mg/kg) and pain killers (buprenorphine 0.2 mg/kg subcutaneously) were given postoperatively three times a day for the total duration of three days. Three weeks after the surgery (study week 3, i.e. preceding the treatments), the rats were divided into groups (Sham, Sham+Oxo, NX, NX+Oxo) so that systolic BPs, body weights and urine volumes were similar in the two Sham and two NX groups. Oxonic acid (Oxo; 20 g/kg chow, Sigma-Aldrich Chemical Co, St Louis, MO, USA) was then added in the chow of the Sham+Oxo and NX+Oxo groups, whereas the Sham and NX groups continued on the normal chow. Hyperuricemia was confirmed by tail vein sampling at study week 5, and 24-hour urine was collected in metabolic cages at the end of the 2nd and 11th study weeks [14]. Before and during the treatment period systolic BP was measured at 28°C by the tail-cuff method as the averages of five recordings in each rat (Model 129 Blood Pressure Meter; IITC Inc., Woodland Hills, CA, USA). The animals were kept in plastic restrainers during the measurements. To increase the reliability, before the actual measurements the rats were preconditioned on two separate occasions.

After 9 weeks of diets, the rats were weighed and anesthetized (urethane 1.3 g/kg) and blood samples from carotid artery for plasma creatinine, urea and UA were drawn in to chilled tubes with EDTA and heparin as anticoagulants. Blood samples from 2 rats in the Sham group were lost due to technical problems. Plasma creatinine and urea were measured using standard clinical chemical methods (Cobas Integra 800 Clinical Chemical Analyzer, Roche Diagnostics, Basel, Switzerland). UA was measured using an enzymatic colorimetric method [18]. The hearts and the kidneys were removed and weighed. The ventricles were snap frozen in liquid nitrogen-cooled isopentane and stored at −70°C until the extraction of the total RNA. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, Finland, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Functional responses and morphology of the mesenteric arterial preparations in vitro

The experiments on isolated arteries were performed from 10 randomly chosen rats in each group. Two successive (3 mm in length) sections from the main branch of superior mesenteric artery were excised beginning 3 mm distally from the mesenteric artery-aorta junction, and small (1.9 mm in length) second order branches were dissected from the mesenteric arterial bed under a microscope (Nikon SMZ-2 T, Nikon Inc., Japan). The endothelium was removed mechanically from the proximal piece of the large artery and from one small arterial ring by perfusing air through the lumen [15,17]. In the distal piece of the main branch and in the other small ring the endothelium was left intact. The large arterial

rings were equilibrated in a resting preload of 4.905 mN/mm [16], while the small arterial preparations were normalized so that the internal diameter of the vessel was set at 90% of that obtained when exposed to intraluminal pressure of 100 mmHg in the relaxed state [15,17]. In the large arteries, the force of contraction was measured with isometric force-displacement transducer and registered on a polygraph (FT 03 transducer, 7E Polygraph; Grass Instrument Co., Quincy, MA, USA) and in the small arteries the computerized Mulvany multimyograph (Model 610A, J.P. Trading, Aarhus, Denmark) was employed [16]. All arterial preparations were kept in physiological salt solution (PSS, pH 7.4) containing (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, and aerated with 95% O_2 and 5% CO_2 at 37°C.

Contractions were cumulatively elicited in response to norepinephrine (NE) and KCl in large and small arterial rings. The main branch of the mesenteric artery was chosen for the relaxation responses due to our extensive previous experience with this model [19-22]. Vasodilatation to Ach was investigated in endothelium-intact rings after precontractions with 5 μM NE in the absence and presence of the non-specific nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0.1 mM). Responses to the NO donor sodium nitroprusside (NP) and BK_{Ca} opener NS-1619 were studied in endothelium-denuded rings precontracted with KCl [23]. The efficiency of the removal of the endothelium was confirmed by the lack of relaxation to Ach [22]. During the experimental protocol, the rings were allowed a 30 min period at baseline tension in between each of the concentration-response challenges.

Morphology of small arteries at 90 mmHg intraluminal pressure was examined using a pressure myograph (Living Systems Instrumentation, Inc., Burlington, VT, USA), as previously reported [24]. The development of myogenic tone was inhibited by Ca^{2+}-free solution containing 30 mmol/L EDTA [25]. Small arteries were chosen for the study of morphology due to their contribution to peripheral vascular resistance via remodeling [26].

Ventricular atrial and B-type natriuretic peptide, skeletal α-actin and ß–myosin heavy chain mRNAs (ANP, BNP, SkαA and β-MHC, respectively)

Total RNA was isolated from the ventricles by the guanidine thiocyanate CsCl method, and 20-μg samples of RNA were transferred to nylon membranes (Osmonics) for Northern blot analysis as described previously [27,28]. Full-length rat ANP cDNA probe (a gift from Dr. Peter Davies, Queen's University, Kingston, Canada), and cDNA probes for rat BNP, SkαA, ß-MHC and 18S were prepared as previously reported [27,28]. The cDNA probes were labeled, the membranes were hybridized and washed, and exposed with PhosphorImager screens (Amersham Biosciences), which were scanned with Molecular Imager FX Pro Plus and quantified using Quantity One software (Bio-Rad) as previously described [27,28]. The hybridization signals of specific mRNAs were normalized to that of 18S RNA in each sample.

Data presentation and analysis of results

Contractile responses were expressed as maximal wall tensions (mN/mm) and as a negative logarithm of the agonist concentration producing 50% of maximal wall tension (pD_2). Relaxations were depicted as percentage of pre-existing contraction. Statistical comparisons were carried out using one-way and two-way analyses of variance (ANOVA), and the Tukey test was used for post-hoc analyses (SPSS 17.0, SPSS Inc., Chicago, IL, USA). ANOVA for repeated measurements was applied when data consisted of repeated observations at successive observation points. The results were expressed as mean ± SEM and $P < 0.05$ denoted statistical significance. Unless otherwise indicated, the *P* values refer to one-way ANOVA.

Drugs

The drugs used in the present study were: ketamine (Parke-Davis Scandinavia AB, Solna, Sweden), cefuroxim, diazepam (Orion Pharma Ltd., Espoo, Finland), metronidazole (B. Braun AG, Melsungen, Germany), buprenorphine (Reckitt & Colman, Hull, England), acetylcholine chloride, norepinephrine bitartrate, L-NAME hydrochloride, NS-1619 (Sigma-Aldrich Chemical Co, St Louis, MO, USA), sodium nitroprusside (Fluka Chemie AG, Buchs SG, Switzerland). Stock solutions of the compounds used in the functional arterial experiments were made by dissolving the compounds in distilled water. Solutions were freshly prepared before use and protected from light.

Results

Blood pressure, body weight, and heart weight

Systolic BP did not differ in the study groups at the beginning of the oxonic acid diet (Table 1). At the end of the study, mean systolic BP was higher in the two NX groups versus Sham groups ($P = 0.041$, two-way ANOVA), and concomitantly increased right and left ventricular weights were also observed. Hyperuricemia did not influence BP or ventricular weights, but was associated with lower final body weight ($P = 0.004$, two-way ANOVA).

Laboratory findings

Plasma UA was 2.4-fold elevated in the Sham+Oxo and 3.6-fold elevated in the NX+Oxo rats when compared with the corresponding controls ($p = 0.005$ and $p = 0.002$, respectively), while subtotal renal ablation did not significantly influence plasma UA levels (Table 1). Plasma

Table 1 Experimental group data (oxonic acid feeding period from week 3 to 12)

	Sham	Sham + Oxo	NX	NX + Oxo
Systolic blood pressure				
Week 3 (before treatment)	119 ± 4	120 ± 5	128 ± 5	127 ± 5
Week 12	133 ± 7	135 ± 5	141 ± 6‡	151 ± 5‡
Body weight (g)				
Week 3	341 ± 6	337 ± 8	335 ± 8	331 ± 7
Week 12	436 ± 9	411 ± 12$^{\#}$	452 ± 11	412 ± 30$^{\#}$
Heart weight (g/kg) at the end of study				
Right ventricle	0.28 ± 0.01	0.29 ± 0.01	0.34 ± 0.03‡	0.33 ± 0.03‡
Left ventricle	1.71 ± 0.06	1.86 ± 0.06	2.23 ± 0.11*‡	2.41 ± 0.18*‡
Removed kidney tissue (g/kg)			7.67 ± 0.17	7.50 ± 0.08
Uric acid (μmol/l)	33.5 ± 11.8	121.6 ± 22.4*	64.4 ± 21.0	156.7 ± 20.2*†
Creatinine (μmol/l)	40.1 ± 5.7	49.3 ± 3.2	80.7 ± 3.2*‡	81.8 ± 8.8*‡
Urea (mmol/l)	6.5 ± 0.4	8.3 ± 0.5	12.9 ± 0.6*‡	14.3 ± 2.2*‡

Values are mean ± SEM; $n = 11$, except for laboratory values in the Sham group $n = 9$. *$P < 0.05$ compared with the Sham group, $^{†}P < 0.05$ compared with the NX group using one-way ANOVA; $^{‡}P < 0.05$ NX groups compared with the Sham groups using two-way ANOVA; $^{\#}P < 0.05$ Sham+Oxo and NX+Oxo groups compared with the Sham and NX groups using two-way ANOVA.

creatinine and urea levels were elevated approximately two-fold in the NX groups and were not affected by hyperuricemia.

Ventricular load, as evaluated using ANP, BNP, SkαA and β-MHC mRNA levels

The synthesis of right and left ventricular ANP and BNP mRNAs, and left ventricular SkαA and β-MHC mRNAs, were clearly higher in the NX groups when compared with the Sham groups ($P < 0.05$ for all, two-way ANOVA) (Figure 1). Oxonic acid feeding did not influence the mRNA levels of these genes.

Functional responses in the main branch of the mesenteric artery

Contractile sensitivity to NE was slightly higher in the NX groups when compared with the two Sham groups ($P < 0.001$, two-way ANOVA). However, the difference in the contractions to NE did not curtail the results on vasorelaxation, as the relaxations were elicited after 5 μM NE concentration that induced approximately 70-80% of maximal response in all groups. Responses to KCl were comparable between NX and Sham rats. The relaxation induced by Ach in the main branch of the mesenteric artery was markedly impaired in the NX groups, while experimental hyperuricemia did not influence these responses either in Sham or NX rats ($P = 0.208$ NX versus NX+Oxo groups, ANOVA for repeated measurements) (Figure 2A). Inhibition of NOS with L-NAME equally reduced the relaxation to Ach in the Sham and Sham+Oxo groups, while the response was almost abolished in the NX and NX+Oxo groups (Figure 2B). As displayed by the diminished relaxation to NP, vasorelaxation via cGMP in arterial smooth muscle was reduced in the NX groups when compared with the Sham groups ($P = 0.008$, two-way ANOVA for repeated measurements), whereas oxonic acid feeding did not influence this response (Figure 2C). In contrast, vasorelaxation induced by the BK_{Ca} opener NS-1619 was significantly reduced in NX+Oxo rats when compared with all other groups ($P = 0.032$, ANOVA for repeated measurements) (Figure 2D).

Functional responses and morphology of the small mesenteric artery

In the small arterial rings, maximal wall tension in response to NE was higher in the NX rats than in Sham rats ($P = 0.03$, two-way ANOVA), while the sensitivity to NE (pD_2) was similar in all groups (Table 2). Responses to KCl were comparable between NX and Sham rats also in the small mesenteric artery. Isolated second order mesenteric artery branches from NX rats exhibited hypertrophic remodeling: increased wall thickness, wall to lumen ratio, and wall cross-sectional area without changes in lumen diameter when compared with Sham rats. Experimental hyperuricemia did not significantly influence the structure of small mesenteric artery (Figure 3).

Discussion

Oxonic acid-induced hyperuricemia has been widely studied in recent years, but consensus on the putative harmfulness of increased circulating UA concentration still remains elusive. In this study we employed the 5/6 nephrectomy rat model of CRI to investigate the influence of experimental hyperuricemia on the tone and morphology of the mesenteric artery. The NX rats showed

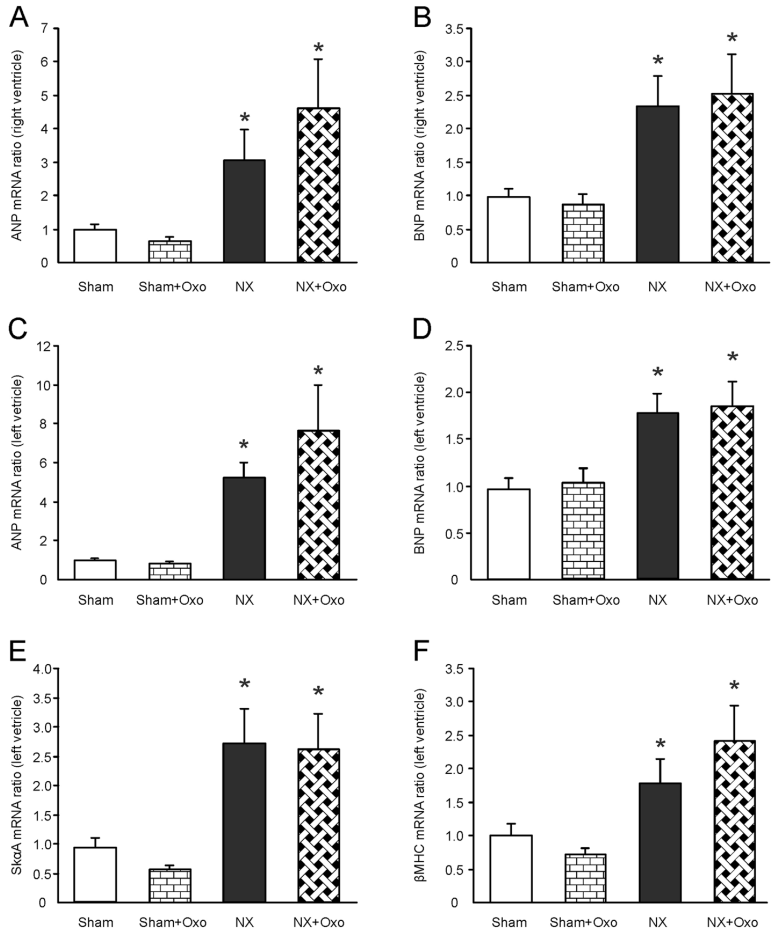

Figure 1 ***Evaluation of cardiac load.*** Right ventricular atrial natriuretic peptide (ANP) **(A)** and B-type natriuretic peptide (BNP) **(B)**; and left ventricular ANP **(C)**, BNP **(D)**, skeletal α-actin (SkαA) **(E)**, and β-myosin heavy chain (β-MHC) **(F)** mRNAs in the experimental groups; values are mean±SEM, $n = 11$ for all groups; *$P < 0.05$, two-way ANOVA compared with the Sham groups. NX=5/6 nephrectomized rat, Sham=sham-operated rat, Oxo=2.0% oxonic acid diet.

several characteristic findings of moderate renal insufficiency [16,29], whereas experimental hyperuricemia did not influence vasoconstrictor responses, renal function, cardiac load, or small artery morphology. To our knowledge, the present study is the first to suggest that hyperuricemia may impair vasorelaxation via BK_{Ca}, indicating alteration in smooth muscle hyperpolarization.

Oxonic acid feeding inhibits the oxidation of UA to its metabolite, allantoin, resulting in hyperuricemia. In the present study, oxonic acid diet elevated plasma UA 2.4 to 3.6-fold, which is in line with the 1.3 to 2.8-fold elevations observed in previous experimental studies [4-6]. The development of stage 2–3 renal insufficiency was confirmed by the elevated levels of plasma creatinine and urea, impaired endothelium-mediated vasodilatation, hypertrophic remodeling of the resistance vessels, and modest elevation of BP [15,29]. Increased right and left ventricular weights, higher ANP and BNP mRNA content, as well as increased left ventricular SkαA and β-MHC mRNA content, indicated permanent volume and pressure overload after subtotal renal ablation [15]. However, hyperuricemia did not significantly influence any of the indicators of cardiac load either in the Sham or NX rats.

Arterial contractions were examined here in order to reveal differences in vasoconstrictor sensitivity that would potentially interfere with the interpretation of the relaxation experiments. No differences were found in responses elicited by membrane depolarisation with KCl

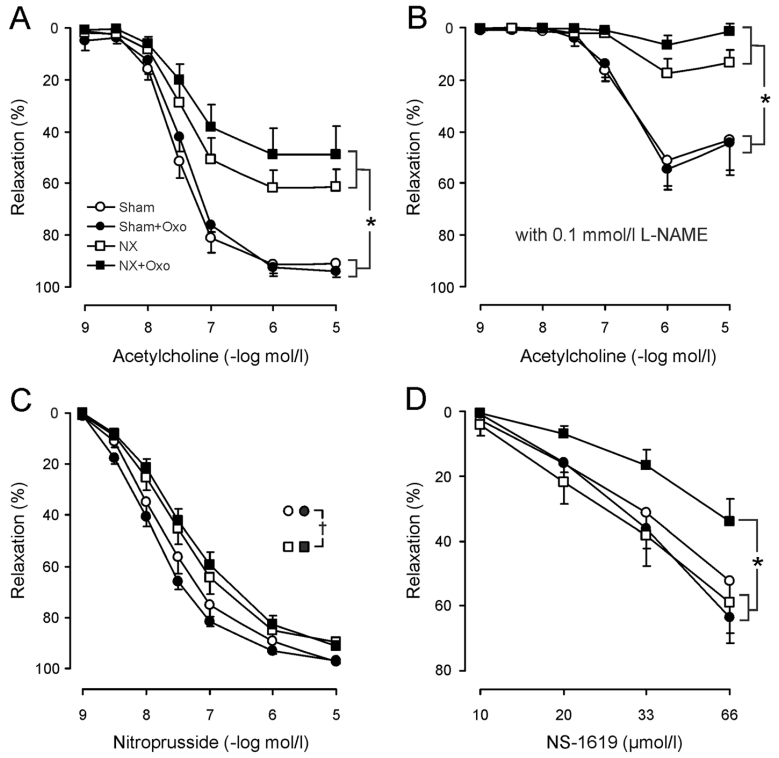

Figure 2 ***Vasorelaxation in vitro.*** Line graphs show relaxation responses induced by acetylcholine in the absence **(A)** and presence **(B)** of L-NAME, and relaxations elicited by the NO donor nitroprusside **(C)**, and the large conductance calcium-activated potassium channel opener NS-1619 **(D)** in the experimental groups; values are mean ± SEM, $n = 10$ for all groups; $^{*}P < 0.05$, ANOVA for repeated measurements; $^{\dagger}P < 0.05$ NX groups compared with the Sham groups using two-way ANOVA for repeated measurements. Groups as in Figure 1.

(Table 2). In the main branch, NX rats exhibited slightly higher sensitivity but no significant change in maximal response to NE. However, in the small artery the NX groups exhibited slightly higher maximal response without changes in sensitivity to NE. Importantly, oxonic acid feeding did not influence the vasoconstrictor responses either in the second order or main branches of the mesenteric artery. Thus, changes in the vasodilator responses induced by hyperuricemia were not explained by alterations in vasoconstrictor responses.

Oxonic acid feeding did not induce changes in the morphology of small mesenteric arteries, while hypertrophic arterial remodeling was clearly observed in the NX rats [15]. Previously, oxonic acid diet was found to activate renal RAS, as indicated by increased expression of juxtaglomerular renin, and result in hypertrophic remodeling of the glomerular afferent arterioles [5-7,30]. Although local vascular RAS components were not examined here, the oxonic acid model of hyperuricemia is characterized by elevated plasma levels of aldosterone with subsequent sodium retention [8]. The present results suggest that despite possible activation of the circulating RAS, high UA level does not influence the morphology of small arteries and heart.

Vasorelaxation was investigated in the main branch of the mesenteric artery, which functionally resembles the second-order branches in the same arterial bed [15,16]. Unlike the rat aorta, where endothelium-dependent vasodilatation is largely mediated via NO, the endothelium-derived relaxation of the mesenteric artery is also mediated by hyperpolarization of smooth muscle [15]. Relaxation to Ach was impaired in NX rats, and the response was practically abolished in the presence of L-NAME in both NX groups, indicating that it was mediated via NO. Inhibition of NOS reduced the relaxation to Ach also in Sham rats, but the vessels showed more pronounced relaxations in the presence of L-NAME than those from NX rats [15,16]. The L-NAME resistant vasorelaxation in the rat mesenteric artery has been attributed to endothelium-dependent hyperpolarization [15-17]. In Sham and NX

Table 2 Parameters of contractile responses of isolated second order branches and the main branch of the mesenteric artery

	Sham	Sham+Oxo	NX	NX+Oxo
Small artery				
Norepinephrine				
pD_2 (–log mol/l)	5.81 ± 0.08	6.00 ± 0.09	5.81 ± 0.09	5.87 ± 0.11
Maximal wall tension (mN/mm)	5.46 ± 0.17	5.82 ± 0.50	6.63 ± 0.54‡	6.58 ± 0.39‡
KCl				
pD_2 (–log mol/l)	1.41 ± 0.01	1.42 ± 0.02	1.39 ± 0.02	1.39 ± 0.03
Maximal wall tension (mN/mm)	5.59 ± 0.43	5.93 ± 0.56	6.37 ± 0.49	6.84 ± 0.32
Main branch				
Norepinephrine				
pD_2 (–log mol/l)	6.26 ± 0.14	5.97 ± 0.05	6.49 ± 0.13‡	6.64 ± 0.11‡
Maximal wall tension (mN/mm)	7.30 ± 0.49	8.60 ± 0.49	9.14 ± 0.63	9.64 ± 1.52
KCl				
pD_2 (–log mol/l)	1.51 ± 0.02	1.51 ± 0.03	1.51 ± 0.03	1.53 ± 0.03
Maximal wall tension (mN/mm)	6.79 ± 0.85	7.01 ± 0.94	7.53 ± 0.67	8.02 ± 0.96

Values are mean ± SEM, $n = 10$ for all groups. pD_2 is the negative logarithm of the concentration of agonist producing 50% of the maximal response. ‡$P < 0.05$ NX groups compared with Sham groups using two-way ANOVA.

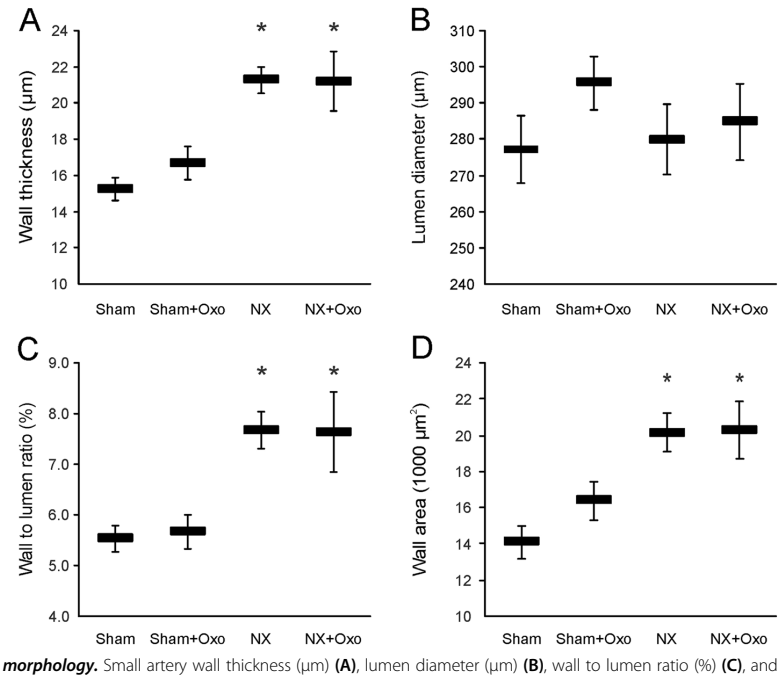

Figure 3 ***Arterial morphology.*** Small artery wall thickness (µm) **(A)**, lumen diameter (µm) **(B)**, wall to lumen ratio (%) **(C)**, and wall area ($µm^2$) **(D)** at 90 mmHg; values are mean ± SEM, $n = 10$ for all groups; *$P < 0.05$ versus Sham. Groups as in Figure 1.

rats hyperuricemia was without significant influence on the response to Ach, indicating that endothelium-dependent relaxation was not affected by oxonic acid feeding. Vasorelaxation to the exogenous NO donor NP was slightly impaired in the NX groups when compared with the Sham groups, but hyperuricemia did not influence the sensitivity of arterial smooth muscle to relaxation via cGMP.

The present study showed that the endothelium-independent vasodilatation induced by the BK_{Ca} channel opener NS-1619 was impaired in the NX+Oxo group. Notably, this impairment was not observed in Sham+Oxo rats. NS-1619 induces relaxation by triggering intracellular Ca^{2+} sparks, which induce K^+-efflux via BK_{Ca} and lead to subsequent hyperpolarization [31-33]. The finding of decreased vasorelaxation sensitivity via BK_{Ca} solely in the hyperuricemic NX rats suggests that the effect was specifically associated with the combination of uremic milieu and increased plasma UA concentration.

Several mechanisms could result in alterations of BK_{Ca} mediated vascular tone. The BK_{Ca} channel, which consists of α- and β1-subunits, is the most prominent type of calcium-activated K^+ channel in arterial smooth muscle [13]. The β1-subunit is responsible for tuning the Ca^{2+}-sensitivity [34]. Interaction between the α-subunit and β1-subunit enhances Ca^{2+}-sensitivity of BK_{Ca} channels, whereas the loss of the β1-subunit decreases Ca^{2+} sensitivity [35]. In a recent study using diabetic mice [36], BK_{Ca} expression in arterial myocytes was strongly influenced by the calcineurin pathway, which inhibits the expression of the regulatory β1-subunit.

Endogenous BK_{Ca} inhibitors may influence K^+-channel activity. Arachidonic acid metabolites, especially 20-hydroxyeicosatetraenoic acid (20-HETE), can inhibit BK_{Ca} [37]. 20-HETE reduces the open-state probability of the channel [38]. Another endogenous inhibitor of BK_{Ca} is hydrogen sulfide, which binds to the α-subunit and increases the voltage needed for channel activation [39]. BK_{Ca} are also modulated by reactive oxidative species (ROS), which can activate or inactivate BK_{Ca} [13]. Such mechanisms are relevant, as elevated UA level following 2.0% oxonic acid feeding has increased total peroxyl radical-trapping capacity and reduced oxidative stress markers in the rat [14]. Hyperuricemia may increase superoxide dismutase (SOD) activity [40], which catalyzes the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (H_2O_2). UA itself is able to scavenge BK_{Ca}-inhibiting radicals, and increase the production of H_2O_2 by preventing the H_2O_2-induced inactivation of SOD [40]. H_2O_2 can even induce vasodilatation directly via BK_{Ca} activation [41], an effect known to be more pronounced under conditions of reduced NO availability [13]. The latter is a characteristic feature of the uremic milieu [42]. Taken together, a multitude of processes can influence vasorelaxation via BK_{Ca}, including changes in channel protein gene expression and structure, changes in cellular Ca^{2+} sparks, levels of ROS, and endogenous BK_{Ca} inhibitors.

UA is produced from xanthine by the enzyme xantine oxidase, which has been found to play an important role in a variety of tissue and vascular injuries [43]. Although therapeutic interventions with the aim to lower UA with xanthine oxidase inhibitors may be beneficial in treating the vascular disorders associated with renal disease, debate is still ongoing whether the effect is related to lowering UA levels *per se*, or to reduced xanthine oxidase activity. The present protocol did not include the treatment of hyperuricemia, since the UA-lowering drugs allopurinol, febuxostat and uricosuric agents have been well documented to prevent the pathophysiological changes induced by the oxonic acid feeding [4-6,10,30,44-46].

Conclusions

We show here that 2.0% oxonic acid diet increased plasma UA, but did not significantly influence BP, resistance vessel structure, and cardiac load as evidenced by the unaltered ventricular weights and mRNA levels of natriuretic peptides, SkαA, and β-MHC. Hyperuricemia did not influence endothelium-dependent NO-mediated vasorelaxation, but oxonic acid feeding impaired vasorelaxation elicited by the BK_{Ca} channel opener NS-1619 in this model of CRI. Future studies are needed to define the molecular mechanisms by which hyperuricemia can influence BK_{Ca} function in experimental CRI.

Abbreviations

20-HETE: 20-hydroxyeicosatetraenoic acid; Ach: Acetylcholine; ANOVA: Analysis of variance; ANP: Atrial natriuretic peptide; BK_{Ca}: Ca^{2+}-activated K^+-channel; β-MHC: ß-myosin heavy chain; BNP: B-type natriuretic peptide; BP: Blood pressure; CRI: Chronic renal insufficiency; L-NAME: N^G-nitro-L-arginine methyl ester; NE: Norepinephrine; NO: Nitric oxide; NOS: Nitric oxide synthase; NP: Nitroprusside; NX: 5/6 nephrectomy; Oxo: 2.0% oxonic acid diet; RAS: Renin-angiotensin system; ROS: Reactive oxygen species; SkαA: Skeletal α-actin; UA: Uric acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VK and TV contributed equally to this article. VK, TV and PP participated in the animal work. VK and TV carried out the *in vitro* vascular function studies, performed statistical analyses, and drafted the manuscript. AE participated in the animal and laboratory work, statistical analyses, and completed the manuscript with IP. JJ carried out the arterial morphology studies. HR and HT were responsible for the natriuretic peptide, as well as α-actin and ß-myosin determinations. ON carried out the blood and urine analyses. JM participated in the study design, financing, and coordination. IP conceived the study design, participated in the animal work and *in vitro* vascular function experiments, financed the study, and completed the manuscript. All authors read and approved the final manuscript.

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PUBLICATION III

Moderate hyperuricemia ameliorated kidney damage in a low-renin model of experimental renal insufficiency. Submitted manuscript.

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Moderate hyperuricaemia ameliorated kidney damage in a low-renin model of experimental renal insufficiency

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Abstract

Uric acid has promoted renal fibrosis and inflammation in experimental studies, but some studies have shown nephroprotective effects due to alleviated oxidative stress. We studied the influence of experimental hyperuricaemia in surgically 5/6 nephrectomized rats. Three weeks after subtotal nephrectomy or sham operation, the rats were allocated to control diet or 2.0% oxonic acid (uricase inhibitor) diet for 9 weeks. Then blood, urine and tissue samples were taken, and renal morphology and oxidative stress were examined. Inflammation and fibrosis were evaluated using immunohistochemistry and real-time PCR (RT-PCR). Remnant kidney rats ingesting normal or oxonic acid diet presented with ~60% reduction of creatinine clearance and suppressed plasma renin activity. Oxonic acid diet increased plasma uric acid levels by >80 μmol/L. In remnant kidney rats, moderate hyperuricaemia decreased glomerulosclerosis, tubulointerstitial damage and kidney mast cell count, without influencing the fibrosis marker collagen I messenger RNA (mRNA) content. In both sham-operated and 5/6 nephrectomized rats, the mast cell product 11-epi-prostaglandin-$F_{2\alpha}$ excretion to the urine and kidney tissue cyclooxygenase-2 (COX-2) levels were decreased. To conclude, hyperuricaemic remnant kidney rats displayed improved kidney morphology and reduced markers of oxidative stress and inflammation. Thus, moderately elevated plasma uric acid had beneficial effects on the kidney in this low-renin model of experimental renal insufficiency.

KEYWORDS
experimental renal insufficiency, hyperuricaemia, kidney morphology, oxonic acid

1 | INTRODUCTION

Hyperuricaemia is a common finding in chronic renal insufficiency (CRI), mainly due to decreased renal uric acid (UA) excretion caused by reduced glomerular filtration and tubulointerstitial damage.[1] Hyperuricaemia may be injurious to the kidneys and the cardiovascular system, but whether hyperuricaemia is a culprit or merely a marker of renal injury remains a matter of debate.[2]

Experimental and clinical studies have linked hyperuricaemia with enhanced cardiovascular complications and progression of CRI. In animal models, oxonic acid (Oxo)-induced hyperuricaemia has been associated with stimulation of the renin–angiotensin system (RAS), endothelial dysfunction, oxidative stress and salt-sensitive hypertension.[3–6] In patients with chronic kidney disease, hyperuricaemia correlated with the severity of glomerulosclerosis and interstitial fibrosis in renal biopsy.[7,8] In contrast, rats receiving intraperitoneal UA infusions presented with antihypertensive and nephroprotective effects, probably due to the antioxidant effects of UA.[9–11] A protective role for UA during acute ischaemic stroke has also been reported.[12–15]

In the absence of previous renal impairment, harmful hyperuricaemia-associated morphological changes have been reported in rat kidneys.[5,16–18] A diet with 2% of the uricase inhibitor Oxo was found to increase collagen deposition, macrophage infiltration, juxtaglomerular renin staining and media–lumen ratio of afferent arterioles and induce glomerular hypertrophy in rat kidneys.[16–18] When Oxo was given daily by gastric gavage to rats for 5 weeks, afferent arteriolopathy and signs of increased intrarenal oxidative stress were observed.[5] In experimental models of CRI, two reports associated hyperuricaemia with harmful effects on the kidneys. In rats subjected to surgical 5/6 nephrectomy (NX), 2% Oxo diet for 6 weeks induced afferent arteriolopathy, enhanced glomerulosclerosis and interstitial fibrosis and increased cyclooxygenase-2 (COX-2) and renin expression in preglomerular vessels.[6] In the ligation type of 5/6 NX (removal of the right kidney and selective ligation of two to three branches of left renal artery), which is a high-renin model of CRI, daily administration of Oxo by gastric gavage for 5 weeks induced renal cortical vasoconstriction and afferent arteriole thickening.[19]

Previously, we found both harmful[20] and beneficial effects[9] in response to hyperuricaemia induced by 2.0% Oxo feeding in rats subjected to surgical 5/6 NX, which is a low-renin model of CRI.[20] Hyperuricaemia increased circulating RAS activity and promoted urinary K^+ loss,[20] but also reduced oxidative stress in vivo as shown by increased plasma antioxidant capacity and decreased urinary 8-iso-prostaglandin-$F_{2\alpha}$ excretion, and improved nitric oxide (NO)-mediated vasorelaxation in the carotid artery.[9] The influences of experimental hyperuricaemia on the kidney and cardiovascular system may depend on the model that was examined, which may partially explain the discrepancies between various studies.[21] These discrepancies may have been due to the differences in modelling schemes between the studies.

In the present study, the hypothesis whether moderate pharmacologically induced experimental hyperuricaemia influences kidney structure was further tested. We determined renal histology and markers of inflammation and fibrosis in 5/6 NX and sham-operated rats that ingested 2.0% Oxo diet for 9 weeks. Before allocation to study groups, our protocol included a 3-week recovery period after the NX surgery to reduce potential selection bias caused by deviations in the degree of renal insufficiency before the 2.0% Oxo diet. In the remnant kidney model of CRI, experimental hyperuricaemia improved kidney morphology, reduced oxidative stress and decreased markers of inflammation.

2 | METHODS

2.1 | Animals and experimental design

Male Sprague–Dawley rats were housed in an animal laboratory with free access to water and chow (Lactamin R34, AnalyCen, Linköping, Sweden) containing 0.9% calcium, 0.8% phosphorus, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 16.5% protein, 4.0% fat, 58% nitrogen-free extract, 3.5% fibre, 6.0% ash, 10% water and 12 550-kJ/kg energy. At the age of 8 weeks (study week 0), the rats were anaesthetized with ketamine (Parke-Davis Scandinavia AB, Solna, Sweden) plus diazepam (Orion Pharma Ltd., Espoo, Finland) using intraperitoneal doses 75 and 2.5 mg/kg, respectively, and NX ($n = 24$) was carried out by the removal of upper and lower poles of the left kidney and the whole right kidney. The kidneys of the Sham rats ($n = 24$) were decapsulated. Anaesthesia, antibiotics and treatment of postoperative pain were as previously reported.[22,23]

Three weeks after the operations, rats were assigned to four groups ($n = 12$ in each): Sham, Sham + Oxo, NX and NX + Oxo. Group sizes were based on our previous experience in this model.[9,20] To ensure corresponding levels of blood pressure (BP) and renal function, the groups were formed so that mean systolic BPs, 24-h urine volumes and body weights in the Sham and Sham + Oxo, and NX and NX + Oxo groups, respectively, were similar. The 2.0% Oxo (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) diet was fed to Sham + Oxo and NX + Oxo groups for 9 weeks, while the Sham and NX groups continued normal diet. Systolic BP was measured at 28°C by the tail-cuff method as the averages of five recordings in each rat (Model 129 BP Meter; IITC Inc., Woodland Hills, CA, USA). The 24-h urine output was collected in metabolic cages at study weeks 3 and 12, and urine samples were stored at −80°C until analyses.

After 9 weeks of the Oxo diet, the rats were anaesthetized with urethane (1.3 g/kg), and blood samples

from cannulated carotid artery were drawn into chilled tubes with heparin or EDTA as anticoagulants. Unexpectedly, cardiac arrest occurred in three Sham group rats, and therefore, no blood samples were obtained from these animals. The kidneys were harvested, kidney halves were frozen in isopentane at −40°C and stored at −80°C. The other kidney halves were fixed in 4% formaldehyde for 24 h and embedded in paraffin. The study was approved by the Animal Experimentation Committee of the University of Tampere and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland (decision LSLH-2003-9718/Ym-23), and conforms to the Guiding Principles for Research Involving Animals. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[24]

2.2 | Blood and plasma determinations

All blood and plasma samples were analysed in a blinded fashion. Plasma creatinine was measured using a standard clinical chemical method (Cobas Integra 800 Clinical Chemical Analyzer, Roche Diagnostics, Basel, Switzerland). UA was measured using an enzymatic colorimetric method[25] and plasma renin activity using a GammaCoat assay (DiaSorin SpA, Saluggia, Italy). All other determinations were carried out as described earlier.[22,23]

2.3 | Kidney morphology and immunohistochemistry

Five-micrometre-thick kidney sections were stained with haematoxylin–eosin, periodic acid Schiff (PAS), toluidine blue or immunohistochemistry and processed for light microscopic evaluation. All kidney morphology analyses were performed by an expert (J.My.) blinded to the groups and treatments.

2.3.1 | Glomerulosclerosis (haematoxylin–eosin and PAS stain)

One hundred glomeruli from each rat were examined at a magnification of ×400 and scored from 0 to 5[26]: 0 = normal; 1 = mesangial expansion or basement membrane thickening; 2 = segmental sclerosis in <25% of the tuft; 3 = segmental sclerosis in 25–50% of the tuft; 4 = diffuse sclerosis in >50% of the tuft; and 5 = diffuse glomerulosclerosis, tuft obliteration and collapse. The damage index for each rat was calculated as a mean of the scores.

2.3.2 | Tubulointerstitial damage (haematoxylin–eosin and PAS stain)

Injury consisting of tubular atrophy, dilatation, casts, interstitial inflammation and fibrosis was assessed in 10 kidney fields at a magnification of ×100.[26] Damage scoring was performed from 0 to 4: 0 = normal; 1 = lesions <25% of the area; 2 = lesions in 25–50% of the area; 3 = lesions in >50% of the area; and 4 = lesions covering the whole area.

2.3.3 | Arteriosclerosis index (PAS stain)

Small arteries were identified from kidney samples magnified ×400 and graded from 0 to 2: 0 = no hyaline thickening; 1 = mild to moderate hyaline thickening in at least one arteriole; and 2 = moderate or severe hyaline thickening in more than one arteriole.[27]

2.3.4 | Kidney mast cells (toluidine blue stain)

Mast cell abundance correlates with renal disease severity.[28] Toluidine blue staining was applied for mast cell identification and quantification. The number of the purple-stained mast cells was counted at a magnification of ×400 and related to kidney tissue area.

2.3.5 | Immunohistochemistry COX-2 and smooth muscle actin (SMA)

Increased COX-2 synthesis has been linked with tissue damage in hyperuricaemia.[6] For the staining of COX-2, a 1:200 dilution of monoclonal anti-COX-2 IgG antibody (RRID AB397602, clone 33, BD Biosciences, San Diego, CA, USA) and, for SMA, a 1:200 dilution of monoclonal anti-SMA IgG antibody (RRID AB2223500, code M0851, Dako Denmark A/S, Glostrup, Denmark) were used. Immunostaining was performed using the Ventana BenchMark LT Automated IHC Stainer (Ventana Medical Systems, AZ, USA) with the ultraView Universal DAB detection kit (catalogue no. 760-500, Ventana Medical Systems) as previously described.[29] The immunohistochemistry staining in kidney tissue was analysed by V.K. and A.E. blinded to the study groups and treatments. Tubulointerstitial

COX-2 staining was scored 0 to 3: 0 = no cells stained; 1 = faint immunoreactivity; 2 = moderate positive staining; and 3 = strong positive staining. Cell positivity (percentage of positive cells) was defined: 0 = no cells stained; 1 = 1–25% positive cells; 2 = 26–75% positive cells; and 3 = >75% positive cells. The results of both analyses were combined for the final score. In the glomeruli, the numbers of COX-2-positive cells were counted and related to tissue area. Staining of SMA was evaluated in an attempt to identify afferent arterioles from the efferent arterioles.[17]

2.4 | Kidney haem oxygenase-1 (HO-1) and collagen I messenger RNA (mRNA) with quantitative real-time PCR (RT-PCR)

Total RNA was isolated from rat kidney tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription of RNA was performed using M-MLV reverse transcriptase (Invitrogen). The expressions of collagen I, oxidative stress indicator HO-1[30–32] and housekeeping control 18S mRNAs were studied by quantitative RT-PCR using ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The following primer sequences were used for collagen I and HO-1: collagen I: forward 5′-TGCAAGAACAGCGTAGCCTACAT-3′ and reverse 5′-AGCGTGCTGTAGGTGAATCGA-3′ (product size 125 bp, accession number NM_053304.1); HO-1: forward 5′-CACAAAGACCAGAGTCCCTCACAG-3′ and reverse 5′-AAATTCCCACTGCCACGGT-3′ (product size 187 bp, accession number NM_012580.2). PCR reactions for collagen I and HO-1 were performed in duplicate in a 25-μl final volume containing 1× SYBR Green Master Mix (Applied Biosystems) and 300 nM of primers. PCR reactions for 18S were performed in duplicate in a 25-μl final volume containing 1× TaqMan Master Mix (Applied Biosystems) and 1× 18S TaqMan Gene Expression Assay primer and probe mix (RRID Hs999999_s1, Applied Biosystems). PCR cycling conditions were 10 min at 95°C and 40 cycles of 20 s at 95°C and 1 min at 60°C. Data were analysed using the absolute standard curve method. 18S was used for normalizing the results as the unnormalized expression of 18S mRNA did not differ between the experimental groups (data not shown).

2.5 | Urine 11-epi-prostaglandin-$F_{2\alpha}$

The concentration of 11-epi-prostaglandin-$F_{2\alpha}$, a mast cell-derived metabolite of prostaglandin D_2 (PGD2),[33,34] was determined from urine collected in metabolic cages after selective solid-phase extraction by radioimmunoassay as previously described.[33]

2.6 | Data presentation and analysis of results

For normally distributed variables, statistical analyses were carried out using one-way and two-way analyses of variance (ANOVAs), as appropriate. For variables with skewed distribution, the Kruskal–Wallis test was applied, with the Mann–Whitney *U* test in the post hoc analyses. Spearman's two-tailed correlation coefficients (r_S) were calculated. Differences between the groups were considered significant when $P < 0.05$, and the Bonferroni correction was applied in the post hoc analyses. The results were expressed as means and standard errors of the mean (S.E.M.s), or as medians, 25th–75th percentiles and ranges. Unless otherwise indicated, the *P* values in the text refer to one-way ANOVA. The statistics were performed using IBM SPSS version 26.0 (Armonk, NY, USA).

3 | RESULTS

3.1 | Animal data

In the beginning of the 2% Oxo diet (study week 3), body weights and systolic BPs were similar in the study groups (Table 1). At study week 12, however, body weights were lower in the NX + Oxo group *versus* NX rats, and two-way ANOVA analysis showed a significant lowering effect of 2% Oxo feeding on body weight ($P = 0.004$). During the follow-up, the two NX rat groups displayed a modest elevation of BP when compared with the two Sham groups (two-way ANOVA $P = 0.041$), while Oxo diet had no statistically significant influence on BP. Urine outputs were similarly higher in both NX groups at study weeks 3 and 12 than in the Sham groups. The surgically removed renal tissue weight was similar in both NX groups. The body weight-adjusted total kidney tissue weight was lower in the NX groups than in the Sham groups, but the remnant left kidney weight in the NX rats was higher than the left kidney weight in the Sham rats (Table 1).

3.2 | Laboratory determinations

The 2% Oxo diet elevated plasma UA levels ~2.5 to 3 times in the NX and Sham rats, respectively (Table 1). Plasma creatinine was elevated by 35–42 μmol/L, and

TABLE 1 Experimental group data and laboratory findings at study weeks 3 and 12

	Sham (n = 9–12)	Sham + Oxo (n = 12)	NX (n = 11–12)	NX + Oxo (n = 12)
Body weight (g)				
Week 3	339 ± 6	338 ± 7	333 ± 8	332 ± 7
Week 12	433 ± 8	412 ± 11[#]	448 ± 10	411 ± 8[†,#]
Systolic blood pressure (mmHg)				
Week 3	120 ± 4	121 ± 5	127 ± 5	125 ± 5
Week 12	134 ± 7	136 ± 5	142 ± 6[‡]	152 ± 4[‡]
Urine volume (ml/24 h)				
Week 3	13.5 ± 0.6	13.8 ± 1.1	31.8 ± 1.8*	31.8 ± 2.6*
Week 12	25.2 ± 1.7	25.8 ± 1.8	53.3 ± 3.8*	49.3 ± 3.9*
Renal tissue removal during 5/6 NX				
Right kidney (g)	n.a.	n.a.	1.53 ± 0.05	1.55 ± 0.04
Left kidney parts (g)	n.a.	n.a.	0.64 ± 0.02	0.61 ± 0.02
Total kidney tissue (g/kg)	n.a.	n.a.	7.67 ± 0.17	7.50 ± 0.089
Final renal tissue weight (g)				
To body weight (g/kg)	6.41 ± 0.18	5.95 ± 0.08	5.24 ± 0.26*	4.97 ± 0.27*
Right kidney (g)	1.30 ± 0.05	1.22 ± 0.03	Removed	Removed
Left kidney (g)	1.37 ± 0.04	1.23 ± 0.03	2.34 ± 0.12*	2.06 ± 0.12*
Laboratory determinations at week 12				
Uric acid (μmol/L)	36 ± 11	117 ± 21*	63 ± 19	152 ± 19*,[†]
Creatinine (μmol/L)	40 ± 5	49 ± 3	82 ± 3*	83 ± 8*
Creatinine clearance (ml/min)	2.9 ± 0.4	2.0 ± 0.2*	1.2 ± 0.1*	1.2 ± 0.1*
Renin activity (ng/ml/h)	27.3 (22.8–30.9)	31.2 (27.8–41.4)	2.0 (0.3–3.1)*	5.0 (2.7–9.9)*
Phosphate (mmol/L)	1.4 ± 0.1	1.5 ± 0.1	1.9 ± 0.2[‡]	1.9 ± 0.2[‡]
Calcium (mmol/L)	2.42 ± 0.02	2.36 ± 0.02	2.42 ± 0.04	2.42 ± 0.02
Sodium (mmol/L)	136.5 ± 0.5	137.3 ± 0.6	136.7 ± 0.9	137.0 ± 0.5
Potassium (mmol/L)	4.1 ± 0.1	3.8 ± 0.1	4.3 ± 0.2	4.4 ± 0.2
Kidney tissue collagen I mRNA copies (× 10^4/ng total RNA)	8.0 ± 0.8	6.6 ± 0.7	11.0 ± 1.9[‡]	10.3 ± 1.9[‡]

Note: Values are mean ± S.E.M. or median (25th–75th percentile).

Abbreviations: n.a., not applicable; NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, sham-operated rat.

*$P < 0.05$ compared with the Sham group.

[†]$P < 0.05$ compared with the NX group.

[#]$P < 0.05$ oxonic acid-treated groups *versus* untreated groups (two-way ANOVA).

[‡]$P < 0.05$ NX groups compared with the Sham groups (two-way ANOVA).

creatinine clearance decreased by ~60% in the NX rats (corresponding to G3b class of decreased glomerular filtration, i.e. moderately to severely decreased renal function[35]), while hyperuricaemia reduced creatinine clearance by ~30% in the Sham rats. Plasma creatinine concentrations in the rats correlated significantly with urine volumes ($r_S = 0.689$, $P < 0.001$). Suppressed plasma renin activity and moderate phosphate retention were observed in both NX groups, whereas the plasma concentrations of calcium, sodium and potassium were corresponding in all groups (Table 1). The 24-h urinary protein excretion was clearly increased in both NX groups (Figure 1A).

3.3 | Renal histology

The indices of arteriosclerosis (Figure 1B), glomerulosclerosis (Figure 1C) and tubulointerstitial damage (Figure 1D) were increased in the NX group, while all

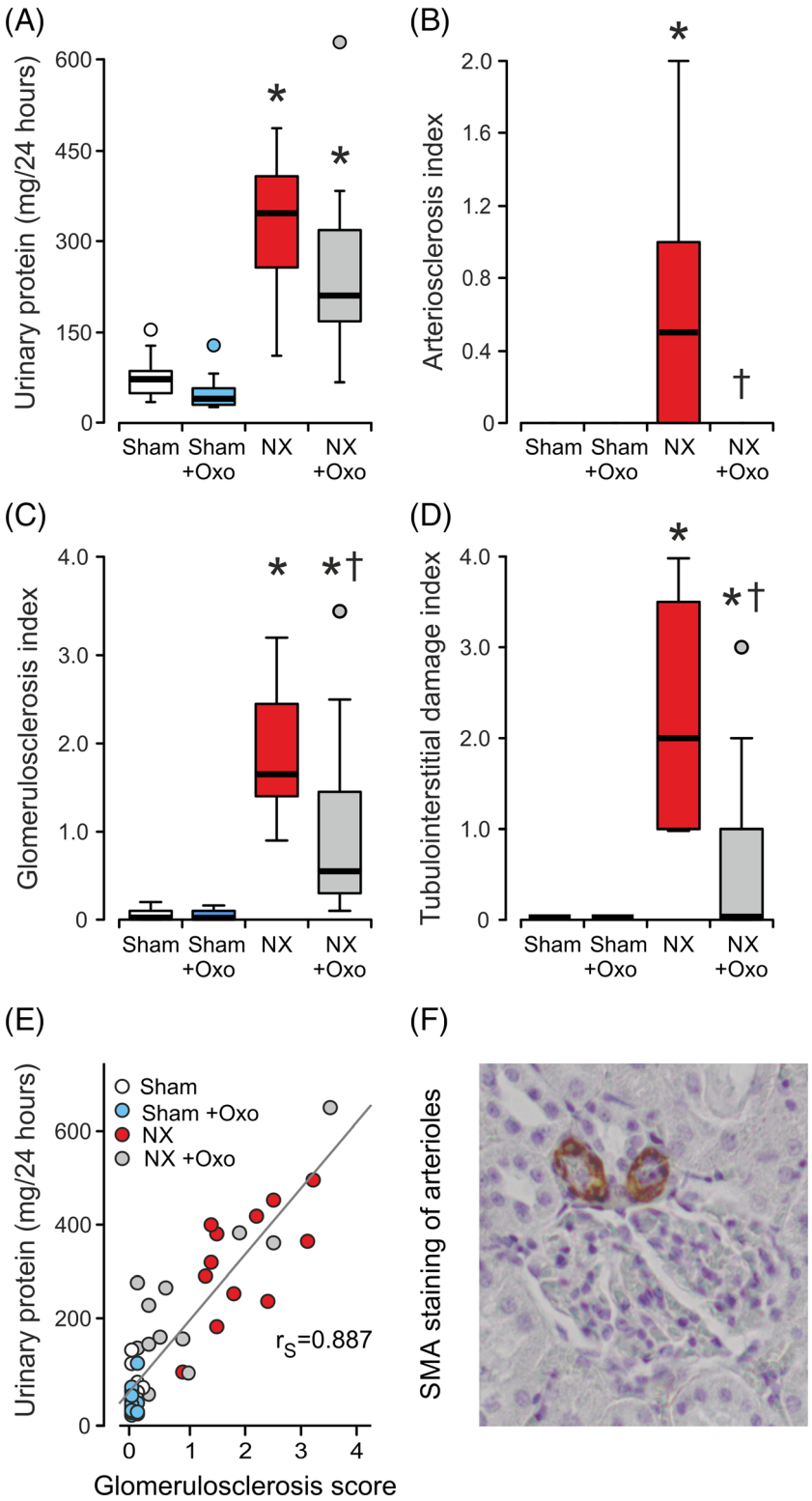

FIGURE 1 Urinary protein excretion during study week 12 (A), kidney arteriosclerosis index (B), glomerulosclerosis index (C), tubulointerstitial damage index (D), correlation between urinary protein excretion and glomerulosclerosis score (E) and representative photomicrograph of smooth muscle actin (SMA) staining of the glomerular arterioles (F) in the study groups ($n = 11–12$ in each group). NX, 5/6 nephrectomized rat; Oxo, 2.0% oxonic acid diet; Sham, sham-operated rat. Values are median (thick line), 25th–75th percentile (box) and range (whiskers), and outliers are depicted as small circles. *$P < 0.05$ *versus* Sham. †$P < 0.05$ *versus* NX

these indices of renal damage were alleviated in the NX + Oxo group when compared with the NX group. The glomerulosclerosis score also significantly correlated with the 24-h urinary protein excretion (Figure 1E).

Staining of SMA was evaluated in an attempt to identify afferent arterioles, as afferent arteriolopathy has been previously reported in a setting where rats were put on 2% Oxo diet immediately after surgical 5/6 NX operation and followed for 6 weeks.[6,17] We observed a clear SMA staining of the arteries that were adjacent to the glomeruli (Figure 1F). However, we could not reliably differentiate the afferent arterioles from the efferent

arterioles with the present techniques. Therefore, further analysis of the preglomerular arterioles was not performed.

3.4 | Markers of inflammation, oxidative stress and collagen I in the kidney

Already 3 weeks after the NX operation when the 2% Oxo diet commenced (study week 3), the 24-h excretion of 11-epi-prostaglandin-$F_{2\alpha}$ to the urine was ~1.6 times higher in the NX groups than in the Sham groups (Figure 2A). At study week 12, the 24-h urinary 11-epi-prostaglandin-$F_{2\alpha}$ excretion was 2.3 times higher in the NX group than in the Sham group, whereas the excretion was reduced by more than 70% in both groups ingesting the 2% Oxo diet (Figure 2B).

Kidney tissue HO-1 mRNA content was higher in both NX groups than in the Sham groups (Figure 2C). The number of mast cells in the kidney tissue was elevated after subtotal NX, while the mast number was significantly lower after the Oxo diet in the remnant kidney rats (Figure 2D). A significant direct correlation between the kidney mast cell count and 24-h urinary 11-epi-prostaglandin-$F_{2\alpha}$ excretion ($r_S = 0.415$, $P = 0.003$) was also observed.

A clear staining of both glomerular and tubulointerstitial COX-2 was observed in the kidney sections (Figure 3A,B). Tubulointerstitial COX-2 score was lower in the Sham + Oxo group than in the Sham group, and in the NX + Oxo group than in the NX group (Figure 3C). The COX-2 staining was abundant in tubuli with thick epithelium corresponding to the ascending limb of the loop of Henle.[36] In the glomeruli, the number of COX-2-positive cells was lower in the Sham + Oxo group than in the Sham group, while lowest COX-2-positive cell numbers were observed in the glomeruli of the NX groups (Figure 3D). The present surgical remnant kidney model was associated with increased kidney tissue collagen I mRNA expression (two-way ANOVA $P = 0.022$), but collagen I mRNA expression was not influenced by the Oxo diet (Table 1).

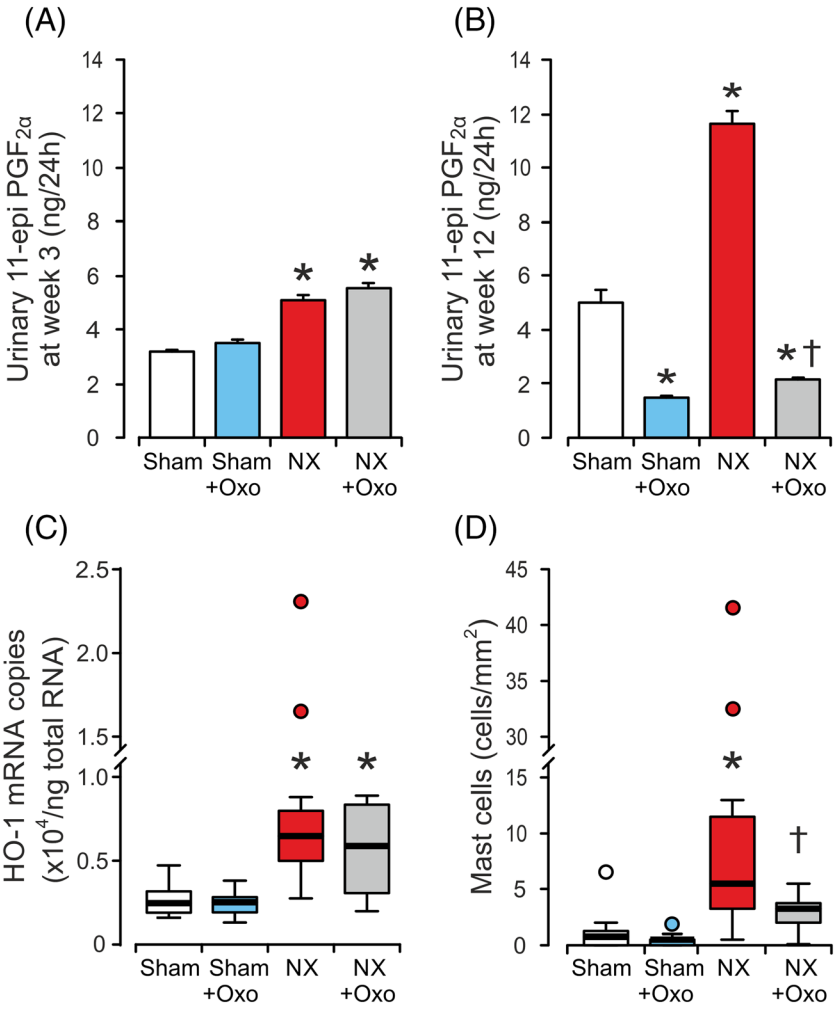

FIGURE 2 The 24-h urinary excretion of 11-epi-prostaglandin $F_{2\alpha}$ during study week 3 (A) and study week 12 (B), kidney haem oxygenase-1 (HO-1) mRNA expression (C) and kidney mast cell content (D) in the study groups ($n = 12$ in each group). Groups as in Figure 1. Values are mean ± S.E.M., median (thick line), 25th–75th percentile (box) and range (whiskers), and outliers are depicted as small circles. *$P < 0.05$ *versus* Sham. †$P < 0.05$ *versus* NX

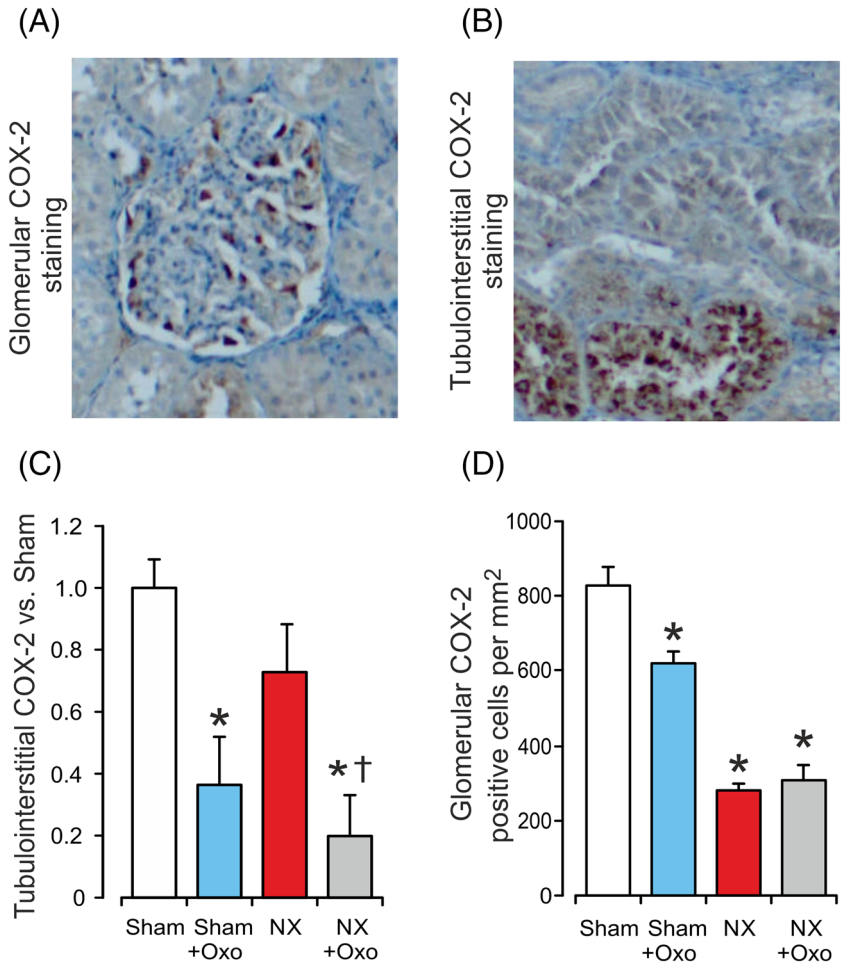

FIGURE 3 Representative photomicrographs of the immunohistochemical staining of glomerular (A) and tubulointerstitial (B) cyclooxygenase-2 (COX-2), tubulointerstitial COX-2 score (C) and number of COX-2-positive cells in the glomeruli (D) in the study groups ($n = 9$–11 in each group). Groups as in Figure 1. Values are mean ± S.E.M. *$P < 0.05$ *versus* Sham. †$P < 0.05$ *versus* NX

4 | DISCUSSION

Here, we examined the effects of 2% Oxo diet-induced hyperuricaemia on kidney morphology, inflammation and markers of oxidative stress in a low-renin model of experimental CRI. Previously, we found that 2% Oxo diet elevated plasma renin and aldosterone, but in parallel improved NO-mediated vasorelaxation in the carotid artery, and reduced oxidative stress in vivo in remnant kidney rats.[9,20] The current results showed that moderately elevated plasma UA level was associated with favourable changes in kidney histology and reduced markers of inflammation in NX rats.

During 2% Oxo feeding, a moderate rise in circulating UA levels is achieved due to inhibition of the hepatic enzyme uricase that metabolizes UA to its final end-product allantoin.[16] Subsequently, plasma UA levels are elevated to concentrations that are closer to those observed in humans. The present 2.5–3 times elevations of UA levels induced by the Oxo diet well correspond to previous findings.[6,16,17,19] Pharmacological lowering of serum UA levels was not included in the present protocol, as several studies have shown that the xanthine oxidase (XO) inhibitors allopurinol and febuxostat effectively prevent the effects of Oxo diet in rats.[5,16,17,19] Notably, the therapeutic effects of XO inhibitors have not been solely related to reduced UA concentrations but also to the anti-oxidative and anti-inflammatory properties of these compounds.[37] The 5/6 NX model has also been characterized by reduced tissue XO activity and a compensatory increase in intestinal UA excretion.[38] These mechanisms may explain why plasma UA was not significantly higher in the NX rats on the normal diet than in Sham rats on the normal diet.

To reduce the risk of selection bias in the current study, the rats were allocated to groups 3 weeks after the operations. The groups were constructed so that BPs, 24-h urine volumes and body weights were similar in the NX and NX + Oxo groups and in the Sham and Sham + Oxo groups, respectively. The 5/6 NX rat model is characterized by immediate and long-term increases in urine volumes,[20,39] probably resulting from high filtration load in the remaining glomeruli and concentrating defect in the remaining nephrons, while the reduction in fractional proximal fluid reabsorption is clearest in the early phase after the renal insult.[39] Of note, in the study by

Kang et al., the division to groups ($n = 4–5$ rats per group) was done immediately after the 5/6 NX operation and was based solely on the amount of the removed kidney tissue.[6] The present renal insufficiency 12 weeks after the NX operation was documented by elevated plasma creatinine, reduced creatinine clearance, hyperphosphataemia and increased urinary protein excretion,[19] while urine volumes were also found to correlate well with the degree of renal insufficiency. The histology showed increased indices of arteriosclerosis, glomerulosclerosis and tubulointerstitial damage in the NX group. The reliability of the histological findings is supported by the good correlation between the glomerulosclerosis score and 24-h urinary protein excretion. Surgical subtotal NX results in marked glomerular hypertrophy so that the size of the glomeruli is increased almost three times when compared with sham-operated controls.[38] The hypertrophy of the remnant kidney can be attributed to compensatory tissue growth in an attempt to compensate for the reduced renal function.[38,40] The NX rats presented with low plasma renin activity probably due to the associated volume load, corresponding to previous findings in rats subjected to surgical renal ablation.[20] Systolic BP was only modestly elevated 12 weeks after renal ablation, as more marked hypertension develops only later in the course impaired renal function in this surgical low-renin model.[41,42]

Previously, the harmful effects of high UA concentrations in renal tissue have been attributed to the deposition of non-soluble monosodium urate crystals in renal tubules (gouty nephropathy).[43] Intracellularly, UA may also mediate biological effects that may play a role in the development of subclinical "non-gouty" types of renal and cardiovascular disease.[43] Excess generation of reactive oxygen species (ROS) has been suggested to play a central role in the UA-induced renal disease.[5] The interaction between UA and ROS is complex, as the synthesis of UA from its purine and pyrimidine nucleotide precursors is catalysed by two xanthine oxidoreductase enzymes: xanthine dehydrogenase and XO. In ischaemic states such as CRI, the latter is the predominant catalyser creating ROS, mainly superoxide anion, as a by-product in the UA synthesis. In cell cultures, UA can inhibit renal production of NO synthase, a catalysing enzyme in NO generation.[16] The reaction between ROS and NO may result in renal NO depletion and afferent artery vasoconstriction, which is an essential step in renal fibrosis.[17] On the other hand, the antioxidant properties of UA are widely accepted. By scavenging superoxide anions, UA can prevent it from reacting with NO and thus inhibit the formation of the toxic peroxynitrite.[10,44] Also, the reaction of UA with peroxynitrite yields a nitrated UA derivate, which has vasodilatory effects.[45] Finally, UA can counter oxidant-induced renal injury by preventing the inactivation of extracellular superoxide dismutase, an enzyme that provides tissue protection by catalysing the dismutation of superoxide radical into oxygen and hydrogen peroxide.[46,47]

We found that the number of mast cells was elevated in remnant kidneys, while mast cell quantity was reduced after the Oxo diet. Kidney mast cell density is known to correlate with the severity of renal disease.[28] Various aetiologies, such as several forms of nephropathies and renovascular ischaemia that cause glomerular damage and interstitial fibrosis, are associated with mast cell abundance in the kidney.[28] Mast cells can aggravate tissue damage and fibrosis by recruiting leucocytes, profibrogenic cytokines, proteases and growth factors and also by directly stimulating collagen synthesis.[28] In an experimental rat model, a close association between mast cell density and oxidative stress in the kidney, as indicated by superoxide anion generation, was previously reported.[48] We also assessed mast cell activity by measuring the quantity of mast cell-derived PGD_2 metabolite, 11-epi-prostaglandin-$F_{2\alpha}$, in the urine.[33,34] Due to the long half-life and stability, 11-epi-prostaglandin-$F_{2\alpha}$ is a convenient way to evaluate mast cell activity in vivo.[33,34,49] The present findings of kidney mast cell density and urinary 24-h 11-epi-prostaglandin-$F_{2\alpha}$ excretion were congruent, and a direct correlation between these variables was observed. Possible explanations to the reduced mast cell infiltration and activity in the renal tissue of hyperuricaemic NX rats are decreased amounts of ROS and increased NO bioavailability,[9] as both of these factors can reduce tissue inflammation and inhibit mast cell degranulation.[48,50]

Whether the actions of UA are detrimental or beneficial may depend on the distribution of UA between the intracellular and extracellular compartments.[43] Extracellularly, the antioxidant properties predominate, whereas intracellularly, UA may be a pro-oxidant.[43] For instance, the free radical scavenging capability of plasma UA appears to have favourable effects on kidney tissue in CRI.[51] In contrast, the blockade of UA entry into the renal tubular cells by the organic anion transporter inhibitor probenecid prevented epithelial-to-mesenchymal transition, an event contributing to progressive tubular fibrosis.[52] We found that kidney tissue HO-1 mRNA content was higher in both NX groups than in the Sham groups but did not differ between the Sham and Sham + Oxo groups, or between the NX and NX + Oxo groups. These findings support the view that the present Oxo diet did not cause oxidative stress even at the cellular level in vivo. Tissue HO-1 content serves as an index of oxidant stress in humans and in animal models of renal disease.[30–32] By converting cell toxic haem to biliverdin in a reaction that liberates carbon monoxide (CO) and iron, HO-1

counteracts the oxidant burden. Inactivation of haem by HO-1 prevents it from inducing lipid peroxidation, and ROS and hydrogen peroxide generation in tubular epithelial cells, while the reaction by-products biliverdin and CO in low concentrations possess antioxidant and vasodilatory effects.[53] In CRI, biliverdin and CO can even help to preserve normal glomerular filtration rate and sodium handling by suppressing tubule-glomerular feedback and afferent arteriolar vasoconstriction.[31]

In the mammalian kidneys, COX-2 has been mainly localized to the macula densa, cortical thick ascending limb and medullary interstitial cells.[36] Increased juxtaglomerular renin and preglomerular arterial COX-2 production have been suggested to contribute to smooth muscle cell proliferation and renal arteriolar obliteration in experimental hyperuricaemia.[6] However, in the present study, the number of glomerular COX-2-positive cells was reduced by 2% Oxo feeding in the Sham rats and was equally further reduced in both NX groups. The explanation for the reduced number of glomerular COX-2-positive cells in both NX groups remains unknown, but may be related to the glomerular hypertrophy and hyperfiltration caused by surgical subtotal NX.[20,38] We found that experimental hyperuricaemia suppressed tubulointerstitial COX-2 protein staining. These findings suggest reduced COX-2-derived inflammatory influences in the kidneys after the 2% Oxo diet. Lower kidney tissue COX-2 content is in line with the beneficial effects of experimental hyperuricaemia on renal histology in the NX rats. Of note, in addition to mast cells, 11-epi-prostaglandin-$F_{2\alpha}$ can also originate from prostanoids synthetized via COX-2. Therefore, reduced 11-epi-prostaglandin-$F_{2\alpha}$ excretion may also reflect reduced total COX-2 content in the kidneys of the hyperuricaemic rats.[54]

Immunohistochemical staining of SMA was done in order to examine the renal preglomerular arterioles, as thickening of the afferent arterioles has been suggested to trigger UA-induced renal fibrosis.[17] The renal arterioles were identified adjacent to glomeruli, but we were unable to reliably differentiate the afferent from the efferent arterioles. Therefore, the present results are inconclusive with respect to preglomerular small artery structure. Increased interstitial collagen deposition has been suggested to mediate UA-mediated renal fibrosis.[16] In the present study, collagen I mRNA expression was elevated in CRI but was not influenced by moderate hyperuricaemia. Although several previous studies have shown that the effects of Oxo feeding are prevented by pharmacological lowering of UA,[5,16,17,19] the possibility remains that some of the present effects were caused by Oxo itself and not by UA.

The causal role of UA in the progression of renal disease has been questioned by Mendelian randomization studies.[55,56] A comprehensive review concluded that the causal association of UA with a range of health outcomes is evident only in gout and nephrolithiasis.[57] In haemodialysis patients, lower UA levels were independently associated with higher all-cause and cardiovascular mortality,[58] while in patients with end-stage renal disease not receiving dialysis or receiving peritoneal dialysis, higher serum UA was associated with higher mortality.[53] There is also evidence that patients genetically predisposed to hypouricaemia present with an elevated risk of renal disease.[59] The optimal range of circulating UA levels in various health conditions warrants further research.

5 | CONCLUSIONS

Consistent with previous reports, the surgical remnant kidney low-renin model of CRI was characterized by renal scarring and increased proteinuria. These pathological alterations were related to increased renal inflammation, fibrosis and oxidative stress, as indicated by increased mast cell infiltration and activation, elevated collagen I mRNA and elevated HO-1 mRNA in the kidneys of the NX rats. Nine weeks of 2% Oxo diet increased plasma UA concentrations and improved renal histology with a parallel reduction in local and urinary markers of inflammation in the remnant kidney rats. These findings indicate that elevated UA levels, which increase the antioxidant capacity in plasma,[9] do not always cause histological and functional impairment of the kidneys.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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PUBLICATION IV

Plasma uric acid is related to large arterial stiffness but not to other hemodynamic variables: a study in 606 normotensive and never-medicated hypertensive subjects

Humam Hamid, Venla Kurra, Manoj Kumar Choudhary, Heidi Bouquin, Onni Niemelä, Mika A.P. Kähönen, Jukka T. Mustonen, Ilkka H. Pörsti and Jenni K. Koskela

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RESEARCH

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Plasma uric acid is related to large arterial stiffness but not to other hemodynamic variables: a study in 606 normotensive and never-medicated hypertensive subjects

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Abstract

Background: Elevated level of plasma uric acid (PUA) has been associated with cardiovascular disease, but whether uric acid is an independent risk factor or merely a marker remains controversial.

Methods: We investigated in a cross-sectional setting the association of PUA with hemodynamics in 606 normotensive and never-medicated hypertensive subjects (295 men, 311 women, age range 19–73 years) without cardiovascular disease or gout. In all except 15 individuals, PUA was within the normal range. Supine hemodynamics were recorded using whole-body impedance cardiography and radial tonometric pulse wave analysis.

Results: The mean concentrations of PUA in age, sex and body mass index adjusted quartiles were 234, 278, 314, and 373 µmol/l, respectively. The highest PUA quartile presented with higher aortic to popliteal pulse wave velocity (PWV) than the lowest quartile (8.7 vs. 8.2 m/s, $p = 0.026$) in analyses additionally adjusted for plasma concentrations of C-reactive protein, low density lipoprotein cholesterol, triglycerides, and mean aortic blood pressure. No differences in radial and aortic blood pressure, wave reflections, heart rate, cardiac output, and systemic vascular resistance were observed between the quartiles. In linear regression analysis, PUA was an independent explanatory factor for PWV ($\beta = 0.168$, $p < 0.001$, R^2 of the model 0.591), but not for systolic or diastolic blood pressure. When the regression analysis was performed separately for men and women, PUA was an independent predictor of PWV in both sexes.

Conclusions: PUA concentration was independently and directly associated with large arterial stiffness in individuals without cardiovascular disease and PUA levels predominantly within the normal range. *Trial registration* ClinicalTrials.gov NCT01742702.

Keywords: Arterial stiffness, Hemodynamics, Impedance cardiography, Pulse wave analysis, Uric acid

Background

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. The World Health Organization estimates that 17.9 million people died from CVDs in 2016, which represents 31% of all global deaths [1]. The role of uric acid, the final product of purine degradation [2], in the development and progression of CVDs has been a subject for an ongoing debate [3, 4].

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An umbrella review analyzing systematic reviews, meta-analyses, and Mendelian randomization studies was recently conducted to investigate uric acid's role in various health outcomes [3]. The outcome was that convincing evidence of a clear role for uric acid existed only regarding nephrolithiasis and gout, whilst suggestive evidence was observed with heart failure, hypertension, impaired fasting glucose or diabetes, chronic kidney disease, and coronary heart disease [3].

One possible link between uric acid and CVD is through arterial stiffness. Decreased large arterial compliance is an acknowledged prognostic marker for CVDs [5, 6]. The recording of pulse wave velocity (PWV) is regarded as the gold standard in the evaluation of large arterial stiffness [7]. In 1225 never-treated hypertensive patients plasma uric acid (PUA) levels were directly and independently associated with carotid-femoral PWV, but negatively associated with augmentation index (AIx), a variable of wave reflections [8]. In the Baltimore Longitudinal Study of Aging, higher serum uric acid was associated with greater increase in PWV in men but not in women during 6 years of follow-up [9]. However, this association was lost when men with PUA $\geq$ 370 µmol/l were excluded, suggesting a threshold for uric acid association with arterial stiffness [9]. A report from the Framingham heart study suggested that every 60 µmol/l increase in uric acid resulted in a 0.04 m/s ($p = 0.016$) increase in carotid-to-femoral PWV when hypertensive subjects were excluded [10]. A recent cross-sectional study found that PWV was higher in medicated hypertensive subjects with hyperuricemia than in hypertensive subjects without hyperuricemia [11]. Also a meta-analysis containing information from 24 publications supported the view that serum uric acid is related to higher carotid-femoral PWV in the general population [4]. However, the matter remains controversial, as serum uric acid was not, but uric acid-to-creatinine ratio in the urine was related to the risk of having higher PWV in 2296 Chinese subjects with a mean age of 43 years [12].

Previously the plasma concentration of uric acid has been related with the level of blood pressure [13], arterial stiffness [4, 9, 11, 14], wave reflections [8, 15], and markers of endothelial injury [16]. In this cross-sectional study our objective was to examine the association of uric acid with several functional hemodynamic variables in a generally healthy population with uric acid concentrations predominantly within the normal range. The present results suggest an independent relation between uric acid and large arterial stiffness, but no association between uric acid and blood pressure (BP), cardiac output, systemic vascular resistance, or wave reflection.

Methods

Study subjects

The recruitment of the study subjects has been previously described [17–19]. All subjects were examined by a physician, office BP was measured, and laboratory analyses were taken as previously described [20]. Laboratory samples were taken 12 (10, 13) days, and medical examination performed 8 (4, 13) days [mean (95% confidence internal, CI)] before the hemodynamic measurements. Medical history, lifestyle behavior and family history were documented. Alcohol use was evaluated as consumption of standard drinks (~ 12 g of absolute alcohol) per week and categorized to low, moderate, high according to the prevailing Finnish guidelines [21]. Smoking amount was estimated in pack-years. All subjects with BP lowering or uric acid level altering medications were excluded. The other exclusion criteria were history of coronary artery disease, stroke, cardiac insufficiency, valvular heart disease, chronic kidney disease, secondary hypertension, alcohol or substance abuse, psychiatric illness other than mild to moderate depression or anxiety, heart rhythm other than sinus rhythm, and ongoing pregnancy. Altogether 606 subjects, aged 20–72 years, participated in the study. The study was registered in two international databases of clinical studies (Eudra-CT registration number 2006-002065-39, ClinicalTrials.gov NCT01742702).

The following stable medications were used by the participants: female hormones for contraception or hormone replacement therapy (n = 63), antidepressants (40), vitamin D supplements (39), hormone-releasing intrauterine devices (24), thyroxin (21), inhaled glucocorticoids (17), antihistamines (16), statins (14), proton pump inhibitors (13), calcium supplementation (10), antirheumatics (6), low dose acetylsalicylic acid (6), anxiolytics (6), inhaled β_2-mimetics (4), non-steroidal anti-inflammatory drugs (3), coxibs (3), pregabalin or gabapentin (3), antiepileptics (2), varenicline (2), warfarin (2), isotretinoin (1), ezetimibe (1), and tramadol (1).

Laboratory analyses

Blood and urine samples were taken after about 12 h of fasting. Plasma C-reactive protein (CRP), sodium, potassium, glucose, creatinine, uric acid, triglyceride, and total, high-density (HDL) and low-density lipoprotein (LDL) cholesterol concentrations were determined using Cobas Integra 800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland). Insulin and parathyroid hormone (PTH) were determined using electrochemiluminescence immunoassay (Cobas e411, Roche Diagnostics). Estimated glomerular filtration rate (eGFR) was calculated using the

CKD-EPI cystatin C equation [22], and insulin sensitivity was evaluated by the quantitative insulin sensitivity check index (QUICKI) [23]. Plasma renin activity (GammaCoat® Plasma Renin Activity 125-I RIA Kit, DiaSorin, Saluggia, Italy) and aldosterone concentration (Active® Aldosterone RIA, Beckman Coulter, Fullerton, CA, USA) were determined using commercial kits. Urine albumin excretion was determined using immunoturbidimetry.

The normal reference range for uric acid in Finland is 230–480 µmol/l for men, 155–350 µmol/l for women aged 18–49 years, and 155–400 µmol/l for women aged ≥ 50 years [24].

Experimental protocol

Hemodynamic recordings were performed in a noiseless, temperature-controlled laboratory [17, 18, 25]. Products containing caffeine, smoking or heavy meal were to be avoided for ≥ 4 h, and alcohol consumption for ≥ 24 h before the investigation. The subjects rested supine on the examination table with impedance cardiography electrodes placed on body surface, tonometric sensor for pulse wave analysis on left radial pulsation, and oscillometric brachial cuff for BP calibration to the right upper arm. The left arm with the tonometric sensor was abducted to 90° in a support, which held the measurement probe at the heart level.

The measurement consisted of one 5-min period with continuous capture of hemodynamic data. For the analyses, the mean values of each 1-min period of recording were calculated. The repeatability and reproducibility of the protocol has been demonstrated to be good [17, 18, 25].

Pulse wave analysis

Radial BP and pulse wave were continuously recorded by a tonometric sensor (Colin BP-508 T, Colin Medical Instruments Corp., USA) [17, 18]. The radial BP was calibrated approximately every 2.5 min by brachial BP measurements. Aortic BP was derived with the validated SphygmoCor pulse wave monitoring system (SpygmoCor PWMx, AtCor medical, Australia) [26]. Aortic pulse pressure and augmentation index (augmented pressure/pulse pressure * 100) were also determined. Central forward wave amplitude was defined as the difference between waveform foot and first systolic inflection point pressure in the aortic waveform [27, 28].

Whole-body impedance cardiography

We used whole-body impedance cardiography (CircMon^R, JR Medical Ltd., Tallinn, Estonia) that records changes in body electrical impedance during cardiac phases to measure heart rate, stroke volume, cardiac output, and PWV [29, 30]. Systemic vascular resistance was calculated from radial BP and cardiac output measured by CircMon^R. Stroke volume, cardiac output and systemic vascular resistance were presented as indexes related to body surface area calculated using the DuBois equation [31]. The method and electrode configuration have been previously reported [30, 32]. Briefly, current electrodes were placed on the distal parts of the extremities proximally to the wrists and the ankles. Voltage electrodes were placed about 5 cm proximally to the current electrodes. To record the distal impedance from the popliteal artery at knee joint level, an active electrode was placed on the lateral side of the knee and the reference electrode on the calf about 20 cm apart. When the pressure wave enters the aortic arch and the diameter of the aorta changes, the voltage electrodes on the distal parts of the extremities record the decrease in whole-body impedance. PWV is calculated from the time difference between the onset of the decrease ('foot') in the whole-body impedance and the popliteal artery signal, and the distance between the electrodes [30, 32].

With CircMon^R the recorded stroke volume and cardiac output are in good agreement with values obtained utilizing 3 dimensional echocardiography [25] and the thermodilution and direct oxygen Fick methods [29, 32], and the PWV values show very good correlation with values measured using ultrasound or the tonometric method [30, 33].

Statistics

The normally distributed data was analyzed using analysis of variance, non-normally distributed data using Kruskal-Wallis test with Mann–Whitney U-test in the post-hoc analyses. The Bonferroni correction was applied in all post-hoc analyses. IBM SPSS Statistics Version 26 (IBM Corporation, Armonk, NY, USA) was used for statistics. The results in the tables were presented as means and standard errors of the mean (SEM), or median [25th–75th percentiles], and in the figures as means and 95% confidence intervals of the mean. $p < 0.05$ was considered significant. The hemodynamic values were calculated as averages from the minutes 3–5 of the recordings when the signal was most stable. For the analyses, the subjects were divided into quartiles of PUA concentration that were adjusted for sex; or age, sex, and body mass index (BMI), as appropriate. Proportions on subjects in the PUA quartiles were compared using the Chi-square test. The use of the medications listed above did not differ between the quartiles of PUA.

For the statistical analyses, the continuous variables not normally distributed were Lg_{10}-transformed. Linear regression analyses with stepwise elimination were used to investigate factors independently associated with hemodynamic variables. The covariates in the

analyses were age, sex, BMI, alcohol consumption category (low, moderate, high) [21], smoking status (never, current, previous); plasma calcium, phosphate, PTH, Lg_{10} of triglycerides, HDL cholesterol, LDL cholesterol, uric acid, renin, aldosterone, Lg_{10} of CRP, sodium; Lg_{10} of QUICKI, eGFR, Lg_{10} of PWV, and mean aortic pressure, as appropriate.

Results

Study population and laboratory values

The PUA concentrations (mean ± SEM) were 344 ± 4 µmol/l in men and 258 ± 3 µmol/l in women ($p < 0.001$). The sex-adjusted demographic characteristics of the study participants according to PUA quartiles are shown in Table 1. Subject age was higher in quartile (Q) 4 versus Q1, while weight was higher in Q3 and Q4 than in Q1 and Q2. No difference was observed in height, whereas BMI was higher in Q3 than in Q2 and Q1 and highest of all in Q4.

Because of the above differences, the laboratory values in Table 2 are presented in sex, age and BMI adjusted quartiles of PUA, the concentrations of which were 234, 278, 314, and 373 µmol/l, respectively. Office systolic BP was higher in Q4 than in Q1, while office diastolic BP was higher in Q3 and Q4 than in Q1. The proportion of subjects with office hypertension (BP ≥ 140/90 mmHg) [20] was higher in Q3 than in Q1. No differences were observed in office heart rate, percentage of smokers, average alcohol intake, plasma electrolyte concentrations, or urine albumin excretion between the quartiles (Table 2). Plasma CRP was slightly higher in Q4 versus Q1 and Q2, while plasma renin activity, and plasma concentrations of aldosterone, PTH, and eGFR were corresponding in all quartiles. No differences were observed in fasting plasma total cholesterol, HDL cholesterol, glucose, and insulin, while QUICKI was lower in Q4 than in Q1. Plasma triglyceride concentration was higher in Q4 and Q3 when compared with Q1, and in Q4 versus Q2, whilst LDL cholesterol was somewhat higher in Q3 than in Q1.

Table 1 Results of the study participants in sex adjusted quartiles of fasting plasma uric acid concentrations

	Q1 n = 141	Q2 n = 155	Q3 n = 165	Q4 n = 144
Male/female (n)	74/77	73/79	76/77	72/78
Age (years)	43.7 (0.96)	44.3 (0.95)	44.3 (0.95)	47.5 (0.96)*
Weight (kg)	74.9 (1.2)	77.5 (1.2)	82.1 (1.2)*†	86.5 (1.2)*†
Height (cm)	173.0 (0.8)	172.9 (0.8)	173.2 (0.8)	172.8 (0.8)
Body mass index (kg/m^2)	24.9 (0.33)	25.7 (0.33)	27.3 (0.32)*†	28.9 (0.33)*†‡

Results shown as mean (standard error of mean)

*$p < 0.05$ versus Q1; †$p < 0.05$ versus Q2; ‡$p < 0.05$ versus Q3

Hemodynamic measurements

The results representing the hemodynamic variables in the age, sex and BMI adjusted quartiles of PUA are shown in Figs. 1, 2 and 3. No differences were observed in radial BP, calibrated from contralateral brachial BP measurements, or aortic BP between the quartiles (Fig. 1A–D).

Aortic pulse pressure, forward wave amplitude and augmentation index were similar in all quartiles (Fig. 2A–C), but aortic to popliteal PWV was higher in Q4 versus Q3 and Q1 (Fig. 2D). When analyzed separately in men and women, PWV was higher in Q4 versus Q1 in men (Fig. 2E), while in women no significant differences in PWV between the PUA quartiles were detected (Fig. 2F). The Pearson correlation between PUA concentration and PWV was 0.351 among all study subjects ($p < 0.001$), 0.338 in women ($p < 0.001$), and 0.242 in men ($p < 0.001$).

No differences were found between the quartiles in heart rate, stroke volume, cardiac output, and systemic vascular resistance (Fig. 3A–D).

Hemodynamic variables in linear regression analyses

The results of the linear regression analyses with stepwise elimination are presented in Tables 3 and 4. The independent explanatory factors for aortic systolic ($R^2 = 0.399$) and diastolic BP ($R^2 = 0.350$) were PWV, eGFR, plasma concentrations of calcium and PTH, and QUICKI (Table 3). In addition, LDL cholesterol and triglycerides were independently associated with systolic BP, while male sex and high alcohol consumption category showed independent associations with diastolic BP (Table 3).

The independent explanatory factors for PWV were age, ejection duration, mean aortic pressure, PUA, plasma triglycerides, BMI, and current smoking ($R^2 = 0.591$) (Table 1). If subjects with PUA ≥ 370 µmol/l were excluded from the regression analysis [9], PUA concentration was still associated with PWV in the remaining 507 participants (beta = 0.003, $p = 0.006$). Altogether the present analyses suggested that for every 100 µmol/l increase in PUA, the associated increase in PWV was 0.9 m/s.

The explanatory variables for PWV were then analyzed separately for sexes (Table 4). In women, the explanatory variables for PWV were age, mean aortic pressure, heart rate, plasma triglycerides, and PUA ($R^2 = 0.580$). In men, the explanatory variables for PWV were age, ejection duration, PUA, mean aortic pressure, BMI, and LDL cholesterol ($R^2 = 0.532$).

Table 2 Clinical characteristics and laboratory results of the study participants in age, sex and body mass index adjusted quartiles of fasting plasma uric acid concentrations

	Q1 n = 141	Q2 n = 155	Q3 n = 165	Q4 n = 144
Office measurements				
Systolic BP (mmHg)	136 (1.5)	140 (1.5)	142 (1.5)	143 (1.5)*
Diastolic BP (mmHg)	87 (0.9)	89 (0.9)	90 (0.9)*	91 (0.9)*
Heart rate (bpm)	66.5 (0.8)	67.2 (0.8)	67.7 (0.8)	68.3 (0.9)
Number (%) of participants with BP ≥ 140/90 mmHg	68 (48.2)	92 (59.4)	106 (64.2)*	89 (61.8)
Current smokers (number)	20	18	18	19
Alcohol (standard drinks/week)	2 [0–6]	2 [0–5]	3 [1–5]	3 [1–7]
Uric acid (μmol/l)	234 (4)	278 (4)*	314 (4)*†	373 (4)*†‡
Sodium (mmol/l)	140 (0.2)	141 (0.2)	140 (0.2)	140 (0.2)
Potassium (mmol/l)	3.8 (0.0)	3.8 (0.0)	3.8 (0.0)	3.8 (0.0)
Calcium (mmol/l)	2.29 (0.01)	2.30 (0.01)	2.31 (0.01)	2.32 (0.01)
Phosphate (mmol/l)	0.95 (0.01)	0.97 (0.01)	0.96 (0.01)	0.99 (0.01)
C-reactive protein (mg/l)	0.5 [0.5–1.4]	0.8 [0.5–1.8]	1.0 [0.5–2.1]	1.0 [0.5-2.1]*†
Renin activity (ng Ang I/ml/h)	0.6 [0.3–1.1]	0.7 [0.4–1.2]	0.7 [0.5–1.3]	0.8 [0.5-1.3]
Aldosterone (pmol/l)	422 [292–569]	449 [329–609]	423 [320–572]	461 [338-620]
PTH (pmol/l)	4.32 (0.13)	4.51 (0.13)	4.75 (0.13)	4.66 (0.14)
eGFR (ml/min/1.73 m^2)	102 (1.2)	100 (1.2)	97 (1.2)	98 (1.2)
Albumin excretion (μg/min)[a]	4 [3–5]	4 [3–6]	4 [3–5]	4 [3–5]
Total cholesterol (mmol/l)	5.0 (0.1)	5.0 (0.1)	5.2 (0.1)	5.2 (0.1)
Triglycerides (mmol/l)	0.9 [0.6–1.2]	1.0 [0.7–1.3]	1.1 [0.8–1.5]*	1.2 [0.8–1.8]*†
HDL cholesterol (mmol/l)	1.65 (0.03)	1.56 (0.03)	1.55 (0.03)	1.57 (0.03)
LDL cholesterol (mmol/l)	2.9 (0.1)	3.0 (0.1)	3.2 (0.1)*	3.1 (0.1)
Glucose (mmol/l)	5.4 (0.1)	5.4 (0.1)	5.4 (0.1)	5.5 (0.1)
Insulin (mU/l)	7.2 (1.4)	7.9 (1.4)	10.7 (1.4)	9.8 (1.5)
QUICKI	0.361 [0.342–0.381]	0.359 [0.367–0.376]	0.352 [0.332–0.372]	0.345 [0.324–0.373]*

Results shown as mean (standard error of mean) or median [27th–75th percentile]; *PTH* parathyroid hormone, *eGFR* cystatin C-based estimated glomerular filtration rate (CKD-EPI) [22], *HDL* high density lipoprotein, *LDL* low density lipoprotein, *QUICKI* quantitative insulin sensitivity check index

*$p < 0.05$ versus Q1; †$p < 0.05$ versus Q2; ‡$p < 0.05$ versus Q3

[a] Albumin excretion results were available 114–130 subjects in each quartile

In additional regression analyses, PUA was not an explanatory factor for forward wave amplitude, augmentation index, systemic vascular resistance, stroke volume, heart rate, or cardiac output (data not shown).

Discussion

In this study, we investigated the association of uric acid with several cardiovascular variables in normotensive subjects and in never-medicated hypertensive patients without cardiovascular disease or gout. In analyses adjusted for confounding factors, PUA was significantly associated with PWV but not with any other hemodynamic variables including radial and aortic BP. The linear regression analyses confirmed that PUA was an independent explanatory factor for PWV, an acknowledged marker of large arterial stiffness [7, 34]. Even when subjects with PUA ≥ 370 μmol/l were excluded [9], PUA concentration remained a significant independent variable related with PWV. Of note, PUA levels were predominantly within the normal range in the present population: only 8/295 men and 7/311 women presented with PUA exceeding the current national upper limits of normal values. Increased arterial stiffness is a strong predictor of cardiovascular events [34], and higher uric acid concentration may thus predispose to the future development of CVD. The average PWV values in the present study were within the normal range for subjects with high-normal BP or grade 1 hypertension [35, 36].

Previous studies have linked uric acid with arterial stiffness, but the matter remains controversial [14, 37]. In a cross-sectional study comprising 651 medicated hypertensive patients and normotensive controls, serum uric

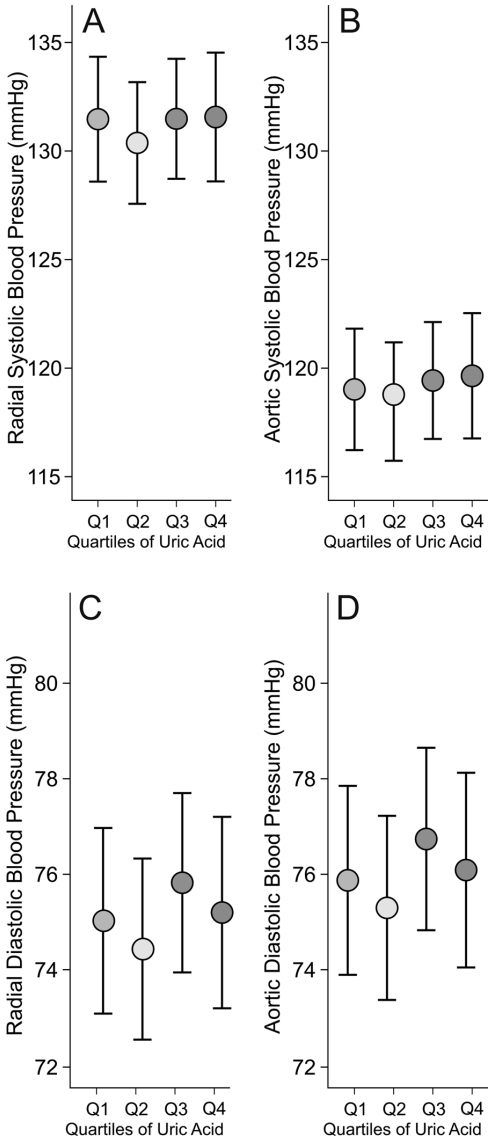

Fig. 1 Radial (**A**) and aortic (**B**) systolic blood pressure, and radial (**C**) and aortic (**D**) diastolic blood pressure in 606 subjects presented in age, body mass index, sex, and plasma C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean

acid was independently associated with carotid-femoral PWV (CF-PWV) [11]. Hypertensive patients with hyperuricemia also presented with higher CF-PWV than hypertensive patients without hyperuricemia [11]. In a large hypertensive population from China comprising 10,450 participants, of whom > 90% were receiving antihypertensive agents and ~ 50% were current smokers, serum uric acid was a risk factor for higher brachial-ankle PWV in men and women [38]. As a major difference to our study, medicated hypertensive subjects were included in the analyses of the above studies [11]. In the Framingham study, Mehta et al. found that uric acid was linked to CF-PWV in subjects with low cardiovascular risk factors, independent of BP [10]. However, when the medicated hypertensive patients were excluded from the analyses, the relation between uric acid and arterial stiffness was much weaker, albeit still statistically significant [10]. In 222 untreated hypertensive subjects without gout, PUA correlated with carotid-femoral PWV but not with 24-h systolic or diastolic BP in univariate analysis, however the association with PWV was no longer significant after correction for albuminuria and other covariates [39]. The above findings suggested that the association of uric acid with large arterial stiffness was not solely explained by the presence of hypertension. Fang et al. examined the relationship of serum uric acid with brachial-ankle PWV in apparently healthy 7025 subjects, and found a significant association in women but not in men [40]. Bian et al. found similar results in their cross-sectional analysis of 2374 subjects, in which elevated uric acid was associated with CF-PWV only in women [15]. On the other hand, in a healthy middle-aged population, serum uric acid was associated with CF-PWV in men but not in women. However, the results were not adjusted for eGFR, insulin resistance, or plasma lipids [41]. In contrast, Cicero et al. reported that uric acid was significantly associated with hypertension and carotid artery intima-media thickness, but not with CF-PWV in 619 subjects not taking antihypertensive, antidiabetic, lipid-lowering and uric-acid-lowering drugs [37].

A possible causal role for uric acid in the development of CVD is still unclear, and no consensus has been reached whether uric acid is a true risk factor or merely a marker of increased risk. However, several mechanisms have been suggested by which hyperuricemia could be linked to cardiovascular pathophysiology. Hyperuricemia may be associated with decreased nitric oxide production, and the resultant endothelial dysfunction could contribute to the development of increased arterial stiffness [16]. Experimental hyperuricemia has been characterized by decreased serum concentration of nitric oxide, while this was reversed by the lowering of uric acid levels using allopurinol [42]. Furthermore, xanthine oxidase inhibition with allopurinol was found to improve endothelium-dependent vasodilatation in the forearm of patients with heart failure and type 2 diabetes [43, 44]. However, the

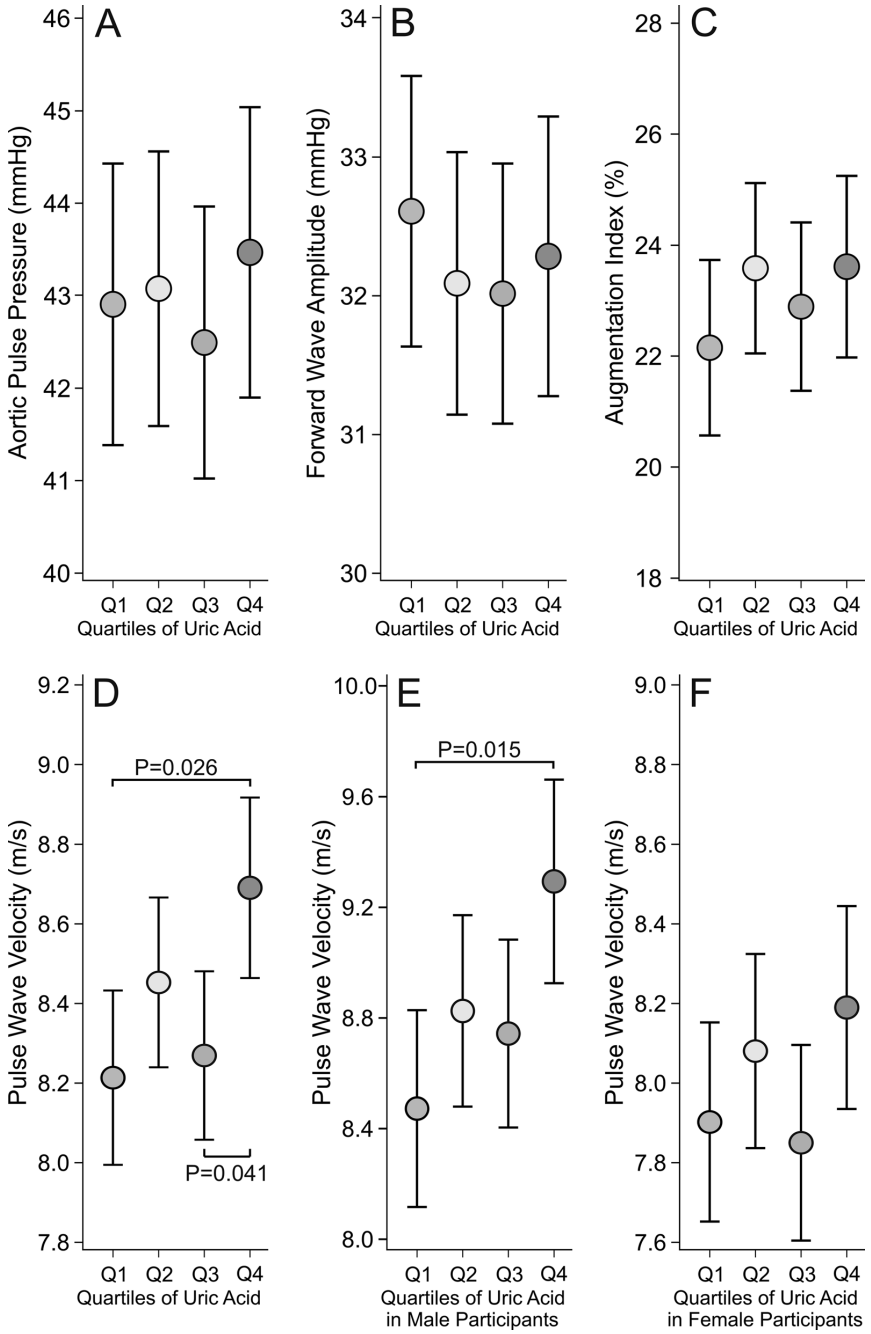

Fig. 2 Aortic pulse pressure (**A**), forward wave amplitude (**B**), augmentation index (**C**), and pulse wave velocity (**D**) in 606 all subjects, and pulse wave velocity separately in men (**E**) and women (**F**), presented in age, body mass index, sex, C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean

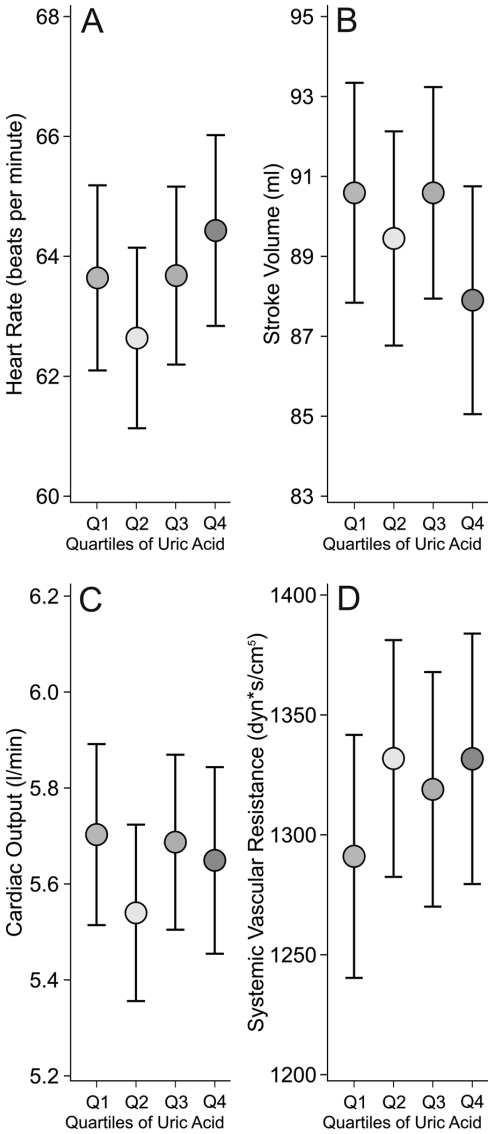

Fig. 3 Heart rate (**A**), stroke volume (**B**), cardiac output (**C**), systemic vascular resistance (**D**) in 606 subjects presented in age, body mass index, sex, C-reactive protein, triglyceride, and LDL cholesterol adjusted quartiles of plasma uric acid concentration; mean ± 95% confidence interval of the mean

interpretation of the above findings is complicated by the potent antioxidant properties of uric acid both in vitro and in vivo [45, 46]. It is therefore possible that the culprit behind impaired endothelial function is the oxidative stress induced by xanthine oxidase activity and not uric acid per se. Indeed, George et al. reported that improved endothelial function induced by allopurinol was due to its ability to reduce vascular oxidative stress and not in its ability to reduce uric acid [47]. Of note, Feig et al. found that treatment of newly diagnosed hypertensive adolescents with allopurinol reduced their BP in the short term [13].

The tonometric recording of PWV is considered the gold standard method for the evaluation of large arterial stiffness [7, 34]. PWV measured by the tonometric recording of arterial pulsations was even found to predict future hypertension in elderly participants of the Framingham study [27]. Aortic-to-popliteal PWV, determined using the same impedance cardiography method that was applied in the present study, was also found to predict future hypertension in young adults [48]. Previously, we reported an excellent correlation ($r = 0.82$) between applanation tonometric measurements of carotid-femoral PWV and impedance cardiography measurements of aortic-to-popliteal PWV in 80 subjects [33]. The impedance-derived PWV has also been validated against the measurement of PWV using ultrasound [30]. Based on the above findings, PWV recorded using whole-body impedance cardiography can be regarded as a reliable measure of large arterial stiffness [30, 33, 48].

We found that office BP was significantly higher in Q4 versus Q1 of PUA, even after adjusting for differences in age, sex, and body mass index (Table 2). However, the present measurements performed in supine position under quiet standardized laboratory conditions did not uncover any significant differences in peripheral or central BP between the PUA quartiles. Previously, white-coat hypertension was reported to be independently associated with higher large arterial stiffness in treated hypertensive patients [49]. Furthermore, when evaluated using the ratio of pulse pressure to stroke volume, higher arterial stiffness was also significantly related to the white-coat effect in 2778 hypertensive patients [50]. As the present quartile with the highest PUA (Q4) also presented with the highest PWV, increased large arterial stiffness may be the plausible explanation for the higher office BP in these subjects versus Q1, because of the link between the white-coat effect and large arterial stiffness [49, 50].

Our study has limitations that need to be acknowledged. PUA and hemodynamics were recorded at single points of time about 12 (10, 13) days apart (mean (95% CI)), and the cross-sectional design does not allow conclusions about causality. We cannot rule out a selection bias caused by the recruitment and exclusion protocol. We applied indirect non-invasive methods requiring mathematical processing to derive PWV, stroke volume

Table 3 Significant explanatory variables for aortic systolic and diastolic blood pressure in linear regression analysis with stepwise elimination

Systolic blood pressure (mmHg)	B	Beta	*p*	Diastolic blood pressure (mmHg)	B	Beta	*p*
$R^2 = 0.399$				$R^2 = 0.350$			
(constant)	− 24.905			(constant)	− 23.172		
Lg_{10} of pulse wave velocity	81.312	0.384	< 0.001	Lg_{10} of pulse wave velocity	47.287	0.333	< 0.001
eGFR	− 0.188	− 0.174	< 0.001	Calcium	20.013	0.159	< 0.001
Calcium	23.794	0.127	< 0.001	PTH	1.013	0.128	0.001
PTH	1.248	0.106	0.003	eGFR	− 0.115	− 0.159	< 0.001
LDL	3.173	0.150	< 0.001	Lg_{10} of QUICKI	− 34.464	− 0.127	0.001
Lg_{10} of QUICKI	− 40.982	− 0.102	0.007	High alcohol consumption category	7.565	0.083	0.020
Lg_{10} of triglycerides	− 7.905	− 0.091	0.030	Sex (male)	2.103	0.080	0.029

Variables in Model: Age, sex, body mass index, alcohol consumption category (low, moderate, high), smoking status (current, previous); plasma uric acid, sodium, calcium, phosphate, PTH, HDL cholesterol, LDL cholesterol, eGFR; Lg_{10} of triglycerides, C-reactive protein, renin, aldosterone, QUICKI, pulse wave velocity. *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *QUICKI* quantitative insulin sensitivity check index, *PTH* parathyroid hormone, *eGFR* estimated glomerular filtration rate from plasma cystatin-C using the CKD-EPI formula [15]

Table 4 Significant explanatory variables for aortic to popliteal pulse wave velocity in linear regression analysis with stepwise elimination

Pulse wave velocity (m/s)	B	Beta	*p*
Men and women, $R^2 = 0.591$			
(constant)	7.594		< 0.001
Age	0.076	0.501	< 0.001
Ejection duration	− 0.020	− 0.223	< 0.001
Mean aortic pressure	0.022	0.180	< 0.001
Uric acid	0.004	0.168	< 0.001
Body mass index	0.032	0.077	0.019
Lg_{10} of triglycerides	0.557	0.069	0.034
Current smoking	− 0.363	− 0.067	0.015
Women, $R^2 = 0.580$			
(constant)	− 0.025		
Age	0.073	0.524	< 0.001
Mean aortic pressure	0.023	0.221	< 0.001
Heart rate	0.032	0.187	< 0.001
Lg_{10} of triglycerides	1.309	0.173	< 0.001
Uric acid	0.003	0.090	0.032
Men, $R^2 = 0.532$			
(constant)	7.747		< 0.001
Age	0.084	0.481	< 0.001
Ejection duration	− 0.024	− 0.230	< 0.001
Uric acid	0.006	0.171	< 0.001
Mean aortic pressure	0.025	0.164	0.001
LDL cholesterol	0.255	0.113	0.014

Variables in Model: Age, sex, body mass index, alcohol consumption category (low, moderate, high), smoking status (current, previous), mean aortic pressure, heart rate, ejection duration, eGFR, uric acid, HDL cholesterol, LDL cholesterol; Lg_{10} of triglycerides; Lg_{10} of C-reactive protein, and QUICKI. *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *QUICKI* quantitative insulin sensitivity check index, *eGFR* estimated glomerular filtration rate from plasma cystatin-C using the CKD-EPI formula [22]

and cardiac output from the bioimpedance signal [32], and central aortic BP waveform from applanation tonometry signal [26]. Even though the methods have been validated against direct or invasive measurements [25, 29, 30], the results must be interpreted with caution. However, the approach to examine central hemodynamics, compared to just focusing on radial or brachial artery pressure, may be better related with the level of cardiovascular risk [51, 52]. Lastly, we cannot rule out potential multicollinearity problems that were not controlled for by the statistical methods, as some of the clinical characteristics and biochemical variables are strongly associated with each other.

Conclusions

A direct association between PUA and PWV was observed in 606 normotensive and never-treated hypertensive subjects without cardiovascular disease or gout. The finding that PUA was not significantly associated with any other hemodynamic variable implies that PUA potentially plays a role in the pathogenesis of large arterial stiffness. Prospective longitudinal studies are needed to confirm the present finding.

Abbreviations

BP: Bloodpressure; BMI: Bodymass index; CVD: Cardiovasculardisease; CF-PWV: Carotid-femoralpulse wave velocity; eGFR: Estimatedglomerular filtration rate; HDL: High-densitylipoprotein; LDL: Low-densitylipoprotein; PTH: Parathyroidhormone; PUA: Plasmauric acid; PWV: Pulsewave velocity; QUICKI: Quantitativeinsulin sensitivity check index; Q: Quartile; SEM: Standarderror of the mean.

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Authors' contributions
HH, VK and IP reviewed the literature and performed the statistical analyses. HH, IP and JK wrote the original version of the manuscript. VK, IP and JK carried out the clinical examinations of patients. HH, MKC, HB, MAPK, JM, IP and JK participated in the design of the technical details and setting up of the methodology. ON was responsible for the laboratory analyses. All authors contributed to the interpretation of results, construction of the discussion and editing the manuscript. IP and JK were the responsible persons for designing and conducting the study. All authors take the responsibility for the contents of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Analyses and generated datasets during the current study are not available publicly as our clinical database contains several indirect identifiers and the informed consent obtained does not allow publication of individual patient data. The datasets are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study protocol and all methods conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study protocol and all methods were approved by the Ethics Committee of the Tampere University Hospital (study code R06086M) and the Finnish Medicines Agency (Eudra-CT registration number 2006-002065-39). Signed informed consent was obtained from all participants. All methods and equipment had also been inspected and approved for clinical use at Tampere University hospital.

Consent for publication
Not applicable.

Competing interests
The authors have no potential competing interests associated with this research.

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