Mechanistic Interactions of Uromodulin with the Thick Ascending Limb: Perspectives in Physiology and Hypertension

Short title: Interactions of UMOD in the TAL

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ABSTRACT

Hypertension is a significant risk factor for cardiovascular disease and mortality worldwide. The kidney is a major regulator of blood pressure and electrolyte homeostasis, with monogenic disorders indicating a link between abnormal ion transport and salt-sensitive hypertension. However, the association between salt and hypertension remains controversial. Thus, there is continued interest in deciphering the molecular mechanisms behind these processes. Uromodulin (UMOD) is the most abundant protein in the normal urine and is primarily synthesised by the thick ascending limb epithelial cells of the kidney. Genome-wide association studies have linked common *UMOD* variants with kidney function, susceptibility to chronic kidney disease, and hypertension independent of renal excretory function. This review will discuss and provide predictions on the role of the UMOD protein in renal ion transport and hypertension based on current observational, biochemical, genetic, pharmacological, and clinical evidence.

Keywords: Tamm-Horsfall Protein; blood pressure; thick ascending limb; electrolyte homeostasis; renal physiology.

Abbreviations: NKCC2, Na^+-K^+-$2Cl^-$ cotransporter; ROMK, renal outer medullary potassium channel; CaSR, calcium-sensing receptor, TNFα, tumour necrosis factor-α; HNF1β, hepatocyte nuclear factor 1β; AngII, angiotensin II; PGE_2, prostaglandin E_2; CLDNs, Claudins; COX2, cyclooxygenase-2; UMOD; uromodulin.

INTRODUCTION

Hypertension is a significant risk factor for cardiovascular disease and mortality worldwide [1]. The World Health Organisation estimates high blood pressure is responsible for the deaths of at least nine million people per annum [1]. Over a third of hypertensive patients remain undiagnosed [1]. It is well documented that the kidney plays a key role in the genesis of hypertension [2–4]. The kidney is a major regulator of electrolyte homeostasis. Monogenic hypertensive disorders suggest a link between abnormal ion transport in the kidney and salt-sensitive hypertension, such as Liddle syndrome caused by gain-of-function mutations of the renal epithelial Na^+ channel ENaC [5]. In numerous epidemiologic, clinical, and experimental studies, dietary sodium has been linked to hypertension, with some patients being particularly salt-sensitive [6–12]. However, there are also studies suggesting a less prominent role for salt in hypertension [13]. It is therefore prudent to analyse the mechanisms responsible for salt-sensitive hypertension.

Uromodulin (UMOD), also known as Tamm-Horsfall protein, is a protein primarily synthesized by thick ascending limb (TAL) epithelial cells of the loop of Henle in the kidney [14,15] and to a lesser extent the early part of the distal convoluted tubule (DCT1) [16]. It is the most abundant protein secreted into the urine of healthy individuals [17]. The TAL encompasses a complex network of components that work together to regulate ion homeostasis. The interdependency of this system means UMOD is not solely responsible for any of its functions, but rather acts in conjunction with these other components. In this review we focus on the predicted roles of the UMOD protein in the physiology of ion transport and its contribution to disease pathogenesis in hypertension.

Biology of UMOD: From Structure to Function

The *UMOD* gene consists of around 20kb and is located on chromosome 16p12.3-16p13.11 [18,19]. It comprises 11 exons, of which exons 2-11 are coding, and is highly conserved across multiple species [18,20]. UMOD protein consists of a 640 amino acid composed of an N-terminal signal sequence (SP); 4 epidermal growth factor (EGF)-like domains (2 of which are calcium binding) which function in adhesion and receptor-ligand interactions; a cysteine-rich domain (D8C); a zona pellucida (ZP) domain; a C-terminal glycosylphosphatidylinositol (GPI) anchoring site (S614); and 8 potential N-linked glycosylation sites (Figure 1a) [21]. The SP is cleaved in the endoplasmic reticulum (ER) and UMOD undergoes extensive glycosylation, which accounts for around 30% of its molecular weight, ranging from 80 to 105 kDa [22].The maturation of UMOD continues within the Golgi apparatus, before being sorted to the apical membrane of the TAL cells, facing the lumen of the tubule. The trafficking proteins involved in the transport of UMOD through these cellular compartments are not well understood. The non-muscle myosin II (NM2) motor proteins have been associated with vesicle biogenesis at the Golgi with different isoforms showing unique localization in the tubules [23]. A preliminary study with conditional genetic knockout mice of NM2 isoforms *Myh9* and *Myh10* in the TAL resulted in an initial aberrant localisation of the UMOD protein, followed by a steady reduction in UMOD protein levels [24].

During its maturation and intracellular trafficking, UMOD is maintained in a polymerization-incompetent state by a polymerization-inhibitory motif formed by hydrophobic interactions between the internal hydrophobic patch (IHP) within the ZP linker region and the external hydrophobic patch (EHP) at the C-terminal of UMOD [25,26]. Once proteolytic cleavage has occurred between the ZP domain and EHP, the active, polymerization-competent UMOD is secreted into the urine, whereas the fragment containing the EHP likely remains attached to the membrane via the GPI anchor. It is understood that the enzyme responsible for proteolytic cleavage of UMOD is the transmembrane type II serine protease hepsin [27], which cleaves

at a conserved site contained within residues 586-589 at the C-terminal (Figure 1b). More recently, cryo-electron microscopy studies have offered detailed insights into this process, whereby the UMOD forms a one-start helix with a 180-degree twists between subunits [28,29] The filament core consists of a zigzag structure with modules of 8.5nm in length [29]. Polymerisation involves major conformational changes in the ZP module's interdomain linker region and depends on the interaction of the activated ZP-C end and ZP-N domain of the following subunit in a head-to-tail mechanism [28,30].

UMOD is also secreted into the serum via the basolateral membrane of the TAL [31]. There is a recent increase in interest for serum UMOD in relation to chronic kidney disease (CKD) and cardiovascular disease [32]. Serum UMOD acts as a kidney and systemic oxidative stress inhibitor through inactivation of the TRPM2 channel, expressed in the brain, bone marrow, spleen, heart, liver, pancreas, lung, stomach, intestine, skeletal muscle, adipose, blood vessel and placenta [33,34]. It was found to be inversely proportional to aortic stiffness in type 1 diabetic adolescents [35]. The role of serum UMOD in hypertension is largely unknown and here we focus on the interaction of UMOD with other TAL components. It should be noted that levels of monomeric UMOD in the blood are 100- to 1000-fold lower than in the urine and as a result there is currently a limited understanding of the biochemical properties of circulating UMOD [36]. Serum UMOD is of particular interest as a biomarker and has been a subject of extensive investigation elsewhere [32,36–38].

Despite the plethora of biochemical studies on the urinary UMOD protein, its precise physiological function is not yet clear. It is understood that secretion of urinary UMOD fluctuates considerably, both within and between individuals [37,39]. Studies in conventional knockout mice (*Umod*$^{-/-}$) have determined that UMOD is involved in water homeostasis and urine concentration [40]. It forms a 3D gel-like network and thus has been proposed to prevent water permeability into the TAL by acting as a seal, maintaining counter-current

gradients in the interstitium [20]. The TAL is involved in the reabsorption of 30% of the filtered Na^+, primarily via the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2), diluting the urine and thereby producing a sufficient osmolality gradient for the vasopressin-dependent absorption of water in the collecting duct [41]. Given the negative charge of UMOD within the urine, it may inhibit the formation of kidney stones by inhibiting aggregation of calcium oxalate and calcium phosphate [42]. UMOD has also been implicated in the protection against urinary tract infections [29,43], as well as displaying immunomodulatory properties by inhibiting viral hemagglutination [44] and supressing *in-vitro* T-cell proliferation by binding tumour necrosis factor-α (TNF-α) and interleukin-1 [20].

Historically, the methods by which UMOD has been measured in biological tissues or fluids have varied significantly and need to be considered when interpreting results. Specific antibodies for human urinary UMOD were characterised early on [45] and since then has been used in several studies [46–48].Although, the concentration of UMOD reported varied due to differences in storage and processing of the urine prior to analysis, such as centrifugation, vortexing, choice of diluent, and freezing-thawing, as discussed in more detail by Youhanna *et al.* [49], which have established a golden standard for UMOD ELISA. Also, contemporary studies normalise UMOD levels from spot urine samples to creatinine as to adjust for differences in time of urine collection, urine concentration and urine flow rate [50]. Another method for quantifying UMOD in urine is via high pressure liquid chromatography (HPLC) together with mass spectrometry (MS). UMOD is typically enriched (e.g. with molecular weight cut-off columns [51], salt precipitation [52], diatomaceous earth [53]), before analysis by MS techniques, such as capillary electrophoresis–mass spectrometry (CE-MS) [54–56], liquid chromatography–mass spectrometry (LC–MS) [33,51,57], and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) [58,59]. The UMOD peptides identified by these MS analyses will differ depending on disease type

and are useful in characterising particular cohorts. For instance, peptidomics performed in urine of preeclamptic women found differential expression of UMOD peptides between cases and normotensive control [54], which were verified in an animal study [55] and a separate study in patients with mild and severe preeclampsia [60].

Measuring urinary UMOD is relevant to renal pathophysiology in clinical research. UMOD has been associated with a number of common and rare kidney diseases [20]. Rare mutations in *UMOD* cause a Mendelian disorder, Autosomal Dominant Tubulointerstitial Kidney disease *UMOD*-related (ADTKD-*UMOD*), which leads to CKD [61,62]. ADTKD-*UMOD* includes familial juvenile hyperuricemia nephropathy (FJHN), medullary cystic kidney disease type 2 (MCKD2), GlomeruloCystic Kidney Disease (GCKD), and uromodulin-associated kidney disease (UAKD) [63]. Genome-wide association studies (GWAS) have linked common *UMOD* variants with kidney function and susceptibility to CKD in the general population [64,65]. These variants map to the promoter region of *UMOD* and affect gene expression [66]. There is no linkage disequilibrium with any protein-coding variant [67]. A summary of all the phenotypic associations of variants in and around the *UMOD* genomic locus is provided in Figure 2. Importantly, *UMOD* promoter variants have also been linked to hypertension independent of renal excretory function [66,68]. This was previously reviewed by Padmanabhan *et al.* in greater detail [69].

UMOD AND HYPERTENSION

It is generally well-documented that the TAL is one of the major regulators of blood pressure and contributes to the pathogenesis of hypertension, with recent reviews covering this in greater depth [41]. However, less is known about the role of UMOD in hypertension. GWAS have been and continue to be essential tools for uncovering associations of the *UMOD* gene with the risk of hypertension (Table 1). A GWAS, based on data obtained from hypertensive

patients, identified the minor G allele of a specific single nucleotide polymorphism (SNP) (rs13333226) in the 5' region of *UMOD* to be associated with a lower risk of hypertension and reduced urinary UMOD secretion, as well as a higher estimated glomerular filtration rate (eGFR) [68]. This signal suggests UMOD may have an effect on hypertension via regulation of renal excretion and was present after adjustment for eGFR [68]. GWAS analysis of thers12917707 variant-G>T, which is in a linkage disequilibrium with rs13333226, showed carriers of the minor T-allele associated with GFR but not blood pressure regulation [70]. The biological effect of these variants became clearer from analyses of nephrectomy samples from patients homozygous for either the risk or protective alleles at variants rs12917707 (G and T, respectively) and rs4293393 (T and C, respectively), whereby expression of *UMOD* was twofold higher in patients with risk variants in the *UMOD* promoter [66]. A meta-analysis of urinary UMOD levels in the general population confirmed a strong association of the rs12917707 risk allele (G) with renal function and demonstrated that carriers of this variant had higher urinary UMOD levels compared to those without this allele [71]. Further to this, variants of genes expressed in the TAL (*KCNJ1, SORSL1,* and *CAB3*) also showed a strong association with urinary UMOD [71]. Overall, this suggests that the genetic and biological effects of *UMOD* on blood pressure are complex.

It should be noted that these are separate GWASs and cannot be directly compared due to differences in cohort structure. While useful, GWAS do not prove any underlying molecular mechanisms involved. Further study into said mechanisms is warranted, given that UMOD is the most abundant protein produced in the TAL and several studies have related its expression and secretion levels to renal function and diseases, suggesting it serves a vital function in the kidney. The complex interactions in the TAL point towards a link between salt, UMOD and hypertension, as evidenced by preclinical and clinical studies.

Clinical Studies

UMOD has been associated with hypertension based on risk alleles identified by GWAS. An analysis of the Swiss Kidney Project on Genes and Hypertension (SKIPOGH) cohort by genotype at rs4293393 SNP of *UMOD* was performed to identify differences in 24h urinary UMOD concentrations (mg/g of creatinine) [66]. The homozygous risk variant (TT) showed the highest urinary UMOD secretion compared to the protective variants (CT and CC) [66]. This paralleled the two-fold increase in *UMOD* transcript levels in kidney samples with the risk haplotype [66]. Indeed, patients homozygous for the risk allele showed increased responses to the loop diuretic furosemide in terms of natriuresis and blood pressure [66].

Clinical studies not considering genetic risk factors for hypertension have also made direct correlations between blood pressure and urinary UMOD secretion. A cross-sectional study on random spot urine samples of 943 participants from a Canadian cohort (CARTaGENE) observed that a higher diastolic BP was associated with a lower UMOD secretion [72]. A case-control study of incident CKD cases and matched controls was conducted on the Systolic Blood Pressure Intervention Trial (SPRINT) trial, a randomized controlled trial undertaken in non-diabetic patients with a high cardiovascular risk and a systolic blood pressure (SBP) of ≥130 mmHg that demonstrated a reduction in major cardiovascular events and death from any cause in patients receiving in the intensive SBP therapy arm (targeting a SBP of <120 mmHg) compared to those that received standard therapy (target SBP of < 140 mmHg) [73,74]. After 1 year of treatment, patients in the intensive arm had a lower incidence of new CKD during the trial and decreased UMOD in spot urine samples compared to the standard arm [73]. This would suggest a higher SBP leads to increased UMOD secretion. In a small study of non-diabetic normotensive and hypertensive patients, no significant difference in UMOD levels were found in 24h urine samples [75]. It has previously been shown that urinary UMOD secretion decreases with age in 24h urine samples [39]. However, it has also been observed that 24h urinary UMOD secretion decreases with age in healthy normotensive

subjects but not in hypertensive patients [76]. In elderly hypertensive patients, a high urinary UMOD was observed to be positively correlated with mean arterial blood pressure compared to elderly normotensive patients [76].

A study has shown that there is no difference in 24h urinary UMOD levels between hypertensive and control patients at baseline [77]. When these hypertensive patients received a 10-day treatment with the loop diuretic furosemide, nifedipine or propranolol, a significant increase in urinary UMOD was seen only in the furosemide group [77]. A small population study has previously described an association between UMOD and dietary salt intake [78]. In this study, 12h (overnight) and 24h urine samples were collected from hypertensive patients subjected to a low-salt diet (10 mmol of sodium per day) for one week, before a transition to a high-salt diet (240 mmol of sodium per day) [78]. UMOD levels decreased in 12h urine samples of low-salt diet patients relative to the transition to a high salt diet, where UMOD levels increased. Conversely, the 24h urine samples showed no statistically significant change in urinary UMOD in low-or high-salt diets compared to the baseline [78]. The SBP of these patients decreased on a low-salt diet compared to baseline and the high-salt diet, as would be expected in salt-sensitive hypertension [78]. The inconsistencies between 12h and 24h urine samples may be elucidated by the fact that water intake is lower overnight, which will affect urine concentration and volume [79,80]. More recently, an analysis of the effects of urinary UMOD levels on salt-induced blood pressure changes in 24h urine samples from the SKIPOGH study demonstrated a trend towards higher SBP with higher sodium intake in individuals with a high UMOD abundance [81]. A quantitative proteomics study found increased levels of UMOD in spot urine of hypertensive patients compared to healthy individuals, yet showed no variation when these hypertensive patients were further divided into salt-sensitive and salt-resistant groups [51]. However, regardless of the presence of

hypertension, those patients homozygous for the *UMOD* risk variant PDILT_UMOD_rs4293393 secreted increased levels of urinary UMOD [51].

Taken together, there are large discrepancies between clinical reports implicating UMOD in hypertension (summarised in Table 2). These differences may be explained through the lack of a standardised system for reporting urinary UMOD levels, including whether to adjust to creatinine levels, the type of urine sample collected (spot, 12h, and 24h), and the relevancy of risk alleles. The factors that regulate UMOD secretion in the general population are poorly understood. The relationship between urinary UMOD secretion and eGFR is controversial, with some studies showing positive associations while others do not [82]. A cross-sectional study analysed urinary UMOD levels in two Swiss population cohorts: SKIPOGH and Cohorte Lausannoise (CoLaus) [39]. The SKIPOGH study showed a positive correlation between eGFR and 24h urinary UMOD. The CoLaus study measured spot morning urinary UMOD concentrations adjusted for creatinine clearance also showed a positive correlation between UMOD and eGFR [39]. More precisely, positive association between UMOD and eGFR occurs when eGFR is <90 mL/min per 1.73m^2 [39]. On the other hand, when eGFR is >90 mL/min per 1.73m^2 the levels of urinary UMOD plateau and adopt Michaelis-Menten kinetics, as is the case in healthy individuals [39]. Therefore, associations may be less accurate at higher eGFRs. It also highlights the importance of an appropriate cohort structure in population studies. Noteworthy is that conditions for UMOD sample processing and storage strongly influence UMOD concentrations and therefore should be considered when interpreting results [49]. This is especially important when considering that it is unclear how UMOD was measured in these clinical studies. Current and future studies should be transparent and follow a clear standard with regards to urinary UMOD measurements. Finally, the effect of hypertension on UMOD protein modification is not well-understood. A study of 24h urine samples from hypertensive patients revealed increased oxidative

modification of UMOD compared to healthy controls which was normalised upon vitamin E supplementation [83]. It is likely that UMOD undergoes varying post-translational modifications in different diseases and requires further research.

Pre-Clinical Studies

A large portion of our current knowledge of the role of UMOD in hypertension stems from animal studies. Kidneys of conventional knockout *Umod*$^{-/-}$ mice, generated by homologous recombination of the *Umod* gene in a breeding scheme of 129/sv, C57Bl/6, and Black Swiss mice, do not show any morphological abnormalities [84]. These *Umod*$^{-/-}$ mice possess lower systolic blood pressures (SBP) compared to the wild-type (WT) in normal conditions [85]. Similarly, tissue-specific *Umod* overexpression in TAL increased blood pressure of transgenic FVB mice in a dose-dependent fashion, with significant differences seen as early as 2 months of age [66]. These mice exhibit salt-sensitive hypertension and renal injury at 16 months of age, paralleling the phenotypes observed in elderly patients homozygous for *UMOD* risk variants [66]. This may be explained through sodium transport in the TAL via the NKCC2, which displayed higher levels of activity in TAL cells of tissue-specific *Umod*-overexpressing mice relative to WT controls, while transcript levels of NKCC2 were not different [66]. The latter implies the functional influence of UMOD on blood pressure occurs on a protein interaction level, rather than a genetic level. These mouse models continue to be relevant to the study of UMOD, as they replicate effects seen in patients with UMOD risk variants [66,67].

With regards to the effect of salt, male Sprague-Dawley rats treated with a high-salt diet over 15 days showed increased mRNA and protein levels of UMOD in the kidneys [86]. A more recent study confirmed this, where high salt-exposure over 2 months increased urinary UMOD secretion starting day 7 in WT C57/BL6 mice, after which a further increase was

seen at the 2-month timepoint [87]. This was matched by an increase in their systolic blood pressure. Salt-loading of conventional *Umod*$^{-/-}$ knockout mice resulted in enhanced urinary TNFα levels [85]. Also, it was shown that TNFα can reduce the levels of NKCC2 mRNA in primary TAL cells, which was enhanced by the absence of UMOD [85]. A recent study reported that global hepsin-deficient C57BL/6J mice generated by ENU mutagenesis have increased UMOD accumulation within the TAL at baseline, specifically in the ER, with hyperactivated NKCC2 [87]. When these hepsin-deficient mice were subjected to a high salt diet for 2 months, no increase in urinary UMOD or blood pressure was observed, instead an increase in intracellular UMOD accumulation and greater urinary salt wasting compared to the WT mice. Although poorly understood, dysregulated UMOD secretion and increased ER stress from its accumulation in TAL cells may profoundly influence salt handling and blood pressure regulation in hypertension.

Conditions such as hypertension in pregnancy have not yet been widely studied. We have previously shown that UMOD proteins exist as both polymerisation-competent and incompetent forms in the urine of normotensive Wistar Kyoto (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP), whereby levels of the latter increase during pregnancy in SHRSP [55]. This would suggest that UMOD and its polymerisation has role in hypertensive pregnancy. It is tempting to relate this to the reduced or inhibited activity of hepsin, as cells lacking this protease only release polymerisation-incompetent UMOD [27].

UMOD INTERACTIONS WITH THE MAJOR COMPONENTS OF THE TAL

DIRECT INTERACTIONS

NKCC2

The TAL region is responsible for the uptake of ~30% of Na^+ load in the kidney via NKCC2, while remaining impermeable to water, thereby concentrating the urine (Figure 3) [88]. Both

NKCC2A and NKCC2F isoforms are expressed in apical surface of TAL, controlling influx of Na^+ accompanied by Cl^- and K^+ uptake, as well as showing differences in Cl^- affinity (high and low, respectively). NKCC2 is key to blood pressure control and has been linked to salt-sensitive hypertension as evidenced by biochemical studies of Dahl salt-sensitive rat models [89–92], as well as genetic [93] and clinical [94] studies in humans. This has recently been reviewed in-depth [95]. Loop diuretics can target NKCC2 and thereby increase Na^+ excretion which can also lead to blood pressure reduction [96].

Studies have shown co-localization of UMOD with NKCC2, which indicates a potential molecular interaction between both proteins [97]. Studies with conventional *Umod*$^{-/-}$ knockout mice demonstrated an impaired ability of these animals to concentrate urine and increased expression of NKCC2 [40]. This implicates an adaption to insufficient Na^+ reabsorption as a direct effect of UMOD on NKCC2 function. Specifically, these mice showed an increased abundance of intracellular NKCC2 located in subapical vesicles [98]. This suggests UMOD promotes NKCC2 activity in TAL by influencing its intracellular trafficking. Transgenic FVB mice overexpressing HA-tagged *Umod* in a TAL-specific manner had increased natriuresis and blood pressure reduction in response to the loop diuretic furosemide that specifically targets NKCC2 [66]. This further substantiates the functional relationship between UMOD and NKCC2.

NKKC2 activity is determined by its phosphorylation at threonine and serine residues located on the N-terminal of the protein [99]. These phosphorylation sites are targeted by SPS1-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1) kinases [100]. These are activated during low Cl^- and hypotonic conditions. The upstream With No Lysine kinases (WNK1 and WNK3) are responsible for activation of SPAK and ORS1 [101,102]. Apart from this signalling cascade, PKA and AMPK have also been implicated in NKCC2 phosphorylation [103]. Immunodetection assays of conventional *Umod*$^{-/-}$ knockout

mice revealed reduced NKCC2 phosphorylation [98]. It is possible that the GPI anchor domain of UMOD, commonly found in membrane trafficking proteins, guides NKCC2 to the apical TAL membrane and acts as a scaffold to promote SPAK and OSR1 phosphorylation of the cotransporter. The levels of active SPAK and OSR1 are increased significantly in TAL-specific *Umod* over-expressing FVB mice, suggesting a promoter role of UMOD in this kinase network [66]. The full role of UMOD in the signalling cascades and regulation of NKCC2 is not yet fully understood.

ROMK

The renal outer medullary potassium channel (ROMK), specifically ROMK2 isoform, is located on the apical membrane of the TAL and is critical to K^+ homeostasis in the TAL [104]. It forms two types of K^+ channels, 30pS and 70pS in the TAL [105]. K^+ ions taken up at the apical or basolateral membrane by NKCC2 or Na^+-K^+-ATPase, respectively, are recycled by ROMK back into the tubular lumen. This generates the K^+ conductance for NKCC2 function and the lumen-positive voltage that drives selective paracellular reabsorption of cations by TAL. ROMK channel mutations cause type II Bartter's syndrome in humans and a similar phenotype in conditional ROMK knockout mice, characterised by salt wasting and dehydration [106,107]. Furthermore, studies have revealed an association between polymorphisms in ROMK with a reduction in blood pressure and protection against hypertension by age 60 [108,109].

The most salient regulators of ROMK channel activity are factors influencing its gating and molecular trafficking. ROMK is activated and maintained in a high open probability state by PKA phosphorylation [110] and PIP_2 binding [111]. Although the precise mediators remain uncertain, cell surface expression of ROMK is dependent on phosphorylation of the N-terminal cytoplasmic residue S44 that overrules an ER retention signal and promotes the

apical movement of ROMK [112]. The membrane trafficking machinery involved in this process is not yet identified, however, yeast-2-hybrid, co-immunoprecipitation and colocalization analyses have implicated an interaction between ROMK2 and UMOD [113]. Conventional *Umod*$^{-/-}$ knockout mice exhibited increased vesicular accumulation of ROMK [113]. Both global and tissue-specific ROMK knockout (*Kcnj1*$^{-/-}$) mice and Bartter- type 2 patients with *KCNJ1* inactivating mutations showed defective urinary UMOD secretion [114]. *In-vitro* pharmacological inhibition and deletion of ROMK in TAL led to reduced trafficking to apical membrane and more intracellular accumulation of UMOD [114]. These two studies suggest that the interaction between UMOD and ROMK is essential for their apical transport. Remarkably, *Umod*$^{-/-}$ mice show K^+ wasting when compared to wild-type (WT) [85].

The Calcium Sensing Receptor (CaSR)

The CaSR is a G-protein coupled receptor and has a wide-spread expression pattern along the nephron with highest expression levels in TAL [115,116]. It is located on the basolateral surface of TAL. The majority of calcium is reabsorbed by the cortical TAL, as opposed to the medullary TAL [117]. It is known that increasing dietary calcium lowers blood pressure in hypertension models [118]. Calcimimetics have been shown to decrease blood pressure in spontaneously hypertensive rats, but not normotensive rats [119]. Activation of the CaSR appears to inhibit renin secretion, lowering angiotensin II (AngII) levels and subsequently decreasing blood pressure [120]. Further study is necessary to determine its precise role in blood pressure regulation in TAL.

CaSR signalling has been related to the secretion of UMOD in TAL [121]. More specifically, mice with global activating mutations in *CaSR* (*Casr*$^{Nuf/Nuf}$) showed a lower urinary secretion of UMOD, whereas mice with global inactivating mutations in the receptor (*Casr*$^{BCH002/+}$) had

enhanced UMOD secretion [121]. These results were further supported in CaSR agonist experiments with TAL cells, whereby calindol reduced apical UMOD secretion [121]. The levels of cAMP were decreased in calindol-treated cells, which aligns with the inhibition and active degradation of cAMP-induced by the CaSR, lowering apical UMOD secretion [121]. Alternatively, increasing cAMP levels via 1-desamino-8 D-arginine vasopressin (dDVAP)-induction increased UMOD secretory levels, which is inhibited with a combinatory calindol treatment [121]. This suggests that the CaSR is vital to the regulation of UMOD trafficking in TAL by influencing cAMP signalling. These changes in UMOD protein levels occur in the absence of total mRNA differences, suggestive of a primarily trafficking influence on UMOD by CaSR signalling [121]. The signalling events that occur downstream of cAMP inhibition, especially in terms of their influence of UMOD trafficking, are unknown and require further investigation. The study suggests a possible involvement of PKA [121]. The CaSR employs multiple heterotrimeric G-proteins ($Gi_{/o}$, $G_{q/11}$ and β-arrestin) to mediate its signalling effects, including stimulating intracellular calcium release, activating mitogen-activated protein kinase (MAPK), membrane ruffling, and inhibition of cAMP [122]. It would be interesting to expand the investigation of biased CaSR G-protein signalling in response to various agonists to the trafficking of UMOD**.**

Direct interaction of UMOD with DCT components: TRPM6, TRPV5 and TRPV6

The kidney is a major site for regulated Mg^{2+} homeostasis. Around ~70% of urinary Mg^{2+} is reabsorbed by the TAL and around 10-20% by the proximal tubule [117]. In the TAL Mg^{2+} reabsorption occurs via the paracellular pathway, as described earlier, whereby ROMK and NKCC2 contribute to formation of a positive lumen potential. Increases in dietary magnesium have been reported to lower blood pressure [123]. A low serum magnesium level (hypomagnesemia) has been associated with increased blood pressure and hypertension [124,125].

The urinary magnesium uptake is fine-tuned by the DCT via an active transcellular pathway mediated by the apical epithelial magnesium channel transient receptor potential melastin 6 (TRPM6) mainly expressed in DCT2 [126]. A magnesium-deficient diet increases TRMP6 expression, implicating the role of renal Mg^{2+} homeostasis in blood pressure control and the pathogenesis of hypertension [127]. More recently, it was demonstrated that UMOD is essential to TRPM6 magnesium homeostasis [128]. Secreted UMOD enhances TRPM6 cell surface abundance and current density by physically interacting with the receptor, with conventional *Umod*$^{-/-}$knockout mice showing lower TRPM6 staining in the DCT [128]. In UMOD construct expression assays it was determined that all UMOD domains (EGF-like domain, D8C cysteine-rich sequence, and ZP domain) but no membrane anchoring may be required for TRPM6 up-regulation [128]. Mechanistically, this is due to UMOD inhibition of dynamin-2 dependent endocytosis of TRPM6 in HEK293 cells [128]. These results were further corroborated in WT 129/SeEv mice fed with low-Mg^{2+}, which showed increased urinary UMOD secretion [128].

Transcellular Ca^{2+} reuptake from the urine is handled by the transient receptor potential cation channel subfamily V member 5 and 6 (TRPV5/6), Ca^{2+}-selective channels expressed in the apical membrane of the second part of the DCT (DCT2), and connecting tubules (CNT) [129]. Urinary UMOD synthesised in TAL and DCT1 was reported to increase TRPV5 apical expression on DCT2 [130]. Immunostaining experiments showed a marked reduction in TRPV5 staining in the DCT of conventional *Umod*$^{-/-}$ knockout mice relative to the WT [130]. Furthermore, it is shown that extracellular UMOD upregulates the surface abundance of TRPV5 by decreasing caveolin-mediated endocytosis [130]. It is interesting to note that UMOD appears to affect TRPM6 in the same cell it is synthesised in for DCT1, but in different cells for TRPV5/6 (DCT2), suggesting it has an autocrine/paracrine-like behaviour.

TNFα

The TNFα cytokine exhibits a multitude of functions in the renal system, ranging from proinflammatory and immunoregulatory effects to modulating ion as well as protein transport mechanisms. TNFα is expressed in TAL and has previously been shown to affect ion transport [131]. AngII stimulation of TAL increases TNFα production via AT-1 receptor activation [132]. TNFα appears to have a context-dependent effect on blood pressure regulation. It induces hypertension in some experimental models based on inflammation, however may have protective effects in other models [133].

More precisely, TNFα production in TAL has been associated with CaSR signalling via G_q and G_i proteins, involving increases in intracellular Ca^{2+}, phospholipase C (PLC) activation and subsequent NFAT activation by calcineurin [134,135]. Studies on cardiac myocytes have shown that TNFα expression is most likely induced by a PKC-dependent pathway involving two transcription factors; nuclear factor-κB (NFκB) and activator protein-1 (AP-1) [136].

TNFα was shown to decrease NKCC2 expression and function in global, conventional $TNF^{-/-}$ knockout mice [137]. In contrast, acute TNFα addition to TAL cells increases ROMK channel activity by protein tyrosine phosphatase signalling [138]. It is understood that UMOD directly interacts with TNFα, though the molecular basis of such interaction are yet to be elucidated [139,140]. *Umod*$^{-/-}$ mice have increased levels of urinary TNFα and enhanced downregulation of NKC22mRNA [85]. Studies have shown that NKCC2-dependent activation of NFAT5 is part of a signalling pathway that triggers TNFα production, thereby inhibiting NKCC2 activity by a negative feedback loop [141]. This suggests TNFα mediates Na^+ homeostasis by maintaining NKCC2 function.

HNF1β

A complex transcriptional network underlies control of TAL function. Hepatocyte Nuclear Factor 1β (HNF1β) is a homeodomain-containing transcription factor that binds DNA as a

homodimer to transactivate transcription. *UMOD* gene expression has been shown to be under direct control of the HNF1β transcription factor, whereby kidney-specific conditional *HNF1β* inactivation in mice leads to a decrease in *Umod* expression [142]. HNF1β is one of the most important regulators of transcription in TAL due to its connection with a myriad of genes involved in signalling pathways, receptors and transporters.

Renal *HNF1β* expression was increased in mice fed on a low Mg^{2+} diet [128]. The transepithelial electrical gradient generated by Na^{+}-K^{+}-ATPase is required for Mg^{2+} reabsorption in TAL. Patients with *HNF1β* mutations showed upregulation of *ATP1A1* which encodes the α1-subunit of Na^{+}-K^{+}-ATPase, with no difference in *CLDN16* expression [143]. HNF1β has also been demonstrated to regulate *CaSR, Cldn14, Cldn19, Cldn10b, Cldn3* \ *Cldn14* gene expression in kidney-specific conditional Ksp-cre *Hnf1β* knockout mice and a *Hnf1β* knockdown immortalised mouse TAL cell line [144].

INDIRECT INTERACTIONS

Claudins and Transcellular Cation Transport

Claudins (CLDNs) are the main proteins that comprise cellular tight junctions and are key to paracellular permeability in the nephron. They act primarily as a selective barrier for small ions (i.e. Mg^{2+}, Ca^{2+} and Na^{+}) and are impermeable to water [145]. CLDN16 and CLDN19 are the main isoforms found in TAL, which together form a pore-forming complex [146]. Studies with TAL-specific *Cldn16* and *Cldn19* siRNA knockdown mice demonstrated an increase in Ca^{2+} and Mg^{2+} urine wasting, highlighting the importance of this complex for reabsorption of these ions [147]. Although CLDN16/19 have not been studied in relation to blood pressure directly, their involvement in cation handling and sodium balance would suggest they contribute to the regulation blood pressure to some extent.

An additional isoform, CLDN14, is known to be expressed in TAL [148]. This isoform has been shown to decrease the cation selectivity of the CLDN16/CLDN19 complex by interacting with CLDN16 [148]. Thus, reducing the calcium and magnesium paracellular permeability. Importantly, the expression of CLDN14 is associated with changes in dietary Ca^{2+} [148], whereby a high Ca^{2+} diet can trigger CaSR-calcineurin–nuclear factor of activated T cell (NFAT) signalling which subsequently increases expression of the *Cldn14* gene [149] via suppression of microRNAs (miR-9 and miR-374) [150]. The CaSR also acts to inhibit CLDN16 via PKA phosphorylation [151]. In relation to UMOD, the CaSR was shown to decrease UMOD secretion [121], which would align with the actions of CLDN14 to reduce calcium reabsorption. This is further underlined by the fact that conventional *Umod*$^{-/-}$ knockout mice had greater urinary magnesium and calcium secretion [128]. The precise mechanism behind this relationship is unclear. Further research is required to substantiate these proposed functions of UMOD with relation to claudins.

AngII Receptor

The renin-angiotensin system (RAS) is one of the most prominent control systems for blood pressure and fluid balance [152]. The biological actions of AngII in the TAL are facilitated by basolateral cell surface transmembrane receptors AT_1 and AT_2 [153,154].

AngII inhibits Cl^- reabsorption in isolated rat TAL tubules *in vitro* in microperfusion flux studies, but stimulates transport if treated with noradrenaline or cAMP [155,156]. NKCC2 has been shown to be inhibited by 20-HETE and NO via AngII signalling [157]. However, AngII enhances PKC activity, which activates superoxide production and ultimately stimulates NKCC2 [158,159]. AngII plays an important role in hypertension and TAL physiology, however its effect on urinary UMOD secretion is unknown. ACE inhibitors have been shown to lower UMOD secretion in patients with essential hypertension [160]. A CE-

MS analysis of patients with macroalbuminuria resulted in the detection of two urinary peptides of UMOD (VIDQSRVLNLGPITR and SVIDQSRVLNLGPITR), which occurred at lower levels compared to patients with normoalbuminuria [161]. Moreover, one peptide (SVIDQSRVLNLGPITR) was found to increase in a dose-dependent fashion upon treatment with AngII receptor blocker candesartan [161]. Taken together, it suggests the influence of AngII signalling arm on UMOD secretion is complex and necessitates further research.

Prostaglandins

Prostaglandins are important lipid mediators within the kidney and control many processes within the TAL. The major renal prostaglandin metabolite is prostaglandin E_2 (PGE_2). The COX-2 enzyme is the rate-limiting inducible enzyme within the PGE_2 synthesis pathway and is expressed in the TAL [162]. It is well documented that prostaglandins play an important role in blood pressure regulation [163]. Clinical studies have associated a COX-2-selective inhibition with hypertension, whereby these patients exhibit Na^+ retention [164,165]. This implicates a role of prostaglandins in maintaining sodium homeostasis and normotension.

PGE_2 has chiefly been studied in the context of modulating the effect of vasopressin (AVP). Activation of the $G_\alpha S$ -protein via vasopressin 2 receptor (V2R) causes an increase in cAMP production, ultimately increasing Na^+ absorption by TAL via NKCC2 [162]. However, intracellular PGE_2 production induced by the CaSR-TNFα pathway attenuates NKCC2 activity [135].

Very little is known about the relationship between PGE_2 and UMOD. Levels of urinary UMOD were found to be reduced in hyperprostaglandin E-syndrome patients, a condition where PGE_2 synthesis is markedly stimulated [166]. This would suggest extracellular PGE_2 normally acts to inhibit UMOD secretion. In contrast, global COX-2 deficient mice with lower intracellular PGE_2 displayed lower urinary UMOD secretion [167].

CONCLUSION

Even though our knowledge of UMOD in hypertension has increased significantly, there are still many challenges and unanswered questions. This includes complications provided by discrepancies between genetic and protein studies. Recently, there has been a growing interest in the polymerisation-status of UMOD, as this is key to determining its function [28,29]. Also, there is a lack of understanding of the functions of circulating UMOD [32]. The factors that dictate basolateral versus apical sorting of UMOD will need to be investigated. Often urinary UMOD is used as an index of UMOD secretion. However, this is usually a composite of expression, exocytosis, cleavage and excretion into the urine. In some of the studies where impaired UMOD secretion is discussed, it is not clear that effects on expression, exocytosis, cleavage, and degradation have been excluded. Furthermore, it is still uncertain in what context to consider measuring UMOD in serum or in 24h urine in a clinical setting. Are any normalizations by kidney function or unit of clearance necessary? The relevance of high or low UMOD concentrations, especially regarding high blood pressure, requires more research.

The main UMOD risk variant (allele T at rs4293393) associated with hypertension has a prevalence of ~80% in Africans and Europeans, and >90% in East Asians, inducing an increase in UMOD expression [168]. However, this has a very minimal effect on blood pressure, warranting further study into other levels of UMOD control, such as posttranslational processes. Gene loci identified by GWASs are limited by the small effect size, with each variant identified only partially responsible for risk of the disease [168]. This is to be expected with polygenic diseases, such as hypertension, as they involve multiple pathways [169]. It should be mentioned that this does not hinder the clinical relevance of currently identified *UMOD* risk variants, but rather paves the way for larger-scale multidisciplinary approaches to fully uncover associated pathogenetic mechanisms.

559 The standard animal models used for the study of UMOD include mice, rats, and rabbits.
560 These are key to unravelling the various interactions within TAL that contribute to UMOD
561 function. However, anatomical and physiological differences between species should be
562 considered, especially in the context of translation of any mechanisms into humans, as these
563 may vary considerably. This is especially true for hypertensive pregnancy models, where
564 translatability may be even more limited.

565 Peptidome analyses have revealed a range of UMOD peptides in the urine in various diseases
566 [55–58,170]. UMOD displays significant potential as a biomarker, with studies linking both
567 circulating and urinary levels to multiple outcomes, such as acute kidney injury [171], tubular
568 function [39,172], CKD [32] and cardiovascular events [173]. A clinical trial testing UMOD-
569 NKCC2 interaction is currently underway (ClinicalTrials.gov Identifier: NCT03354897) with
570 results expected in 2021, which will inform whether loop diuretics can be repositioned in the
571 HTN care pathway based on the *UMOD* promoter genotype [174]. Standardised and reliable
572 quantification methods will need to be established before they can be applied clinically.

573 In conclusion, the various interactions formed by UMOD in the TAL warrant further
574 investigation and will help elucidate processes of hypertension, CKD and renal diseases in
575 general. This, in turn, will guide the design of novel, target-specific therapeutics.

576

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CONFLICT OF INTEREST

There are no conflicts of interest.

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Tables

Table 1 – *UMOD* gene variant risk alleles and their frequency in different populations.

UMOD variant	Allele	GWAS Risk	Disease Association	Urinary UMOD levels	Frequency (%) [$]			References
					Africans	Europeans	East-Asians	
rs13333226	A	Risk	Hypertension	High	67	82	93	[68]
	G	Protective		Low	33	18	7	
rs12917707	G	Risk	CKD	High	96	83	99	[64], [71]
	T	Protective		Low	4	17	1	
rs4293393	T	Risk	CKD, Hypertension	High	70-80	70-80	90	[66], [168], [175]
	C	Protective		Low	20-30	20-30	10	

[$] Data for allele frequency sourced from the National Library of Medicine dbSNP database.
UMOD, uromodulin. CKD, chronic kidney disease.

Table 2 – Summary of patterns of urinary UMOD secretion with various non-genetic clinical factors.

Clinical Factors	Urine Sample	Urinary UMOD levels with respect to Controls	References
Age (SKIPOGH study 45±17 years and CoLaus study 53±11 years)	Spot and 24h	Lower	[39]
Hypertension (>60 years)	24h	Higher	[76]
Salt-Sensitive Hypertension vs. Salt-Resistant Hypertension	Spot	No change (non-significant)	[51]
High DBP	Spot	Lower	[72]
Intensive SBP therapy (<120 mmHg) vs. Standard SBP therapy (<140mmHg)	Spot	Lower	[73]
Loop diuretic furosemide	24h	Lower	[77]
High Salt-Intake vs. Low Salt-intake	12h	Higher (high-salt)	[78]
	24h	No change (non-significant)	[78]
High Salt-Intake vs. Low Salt-Intake	24h	Higher (high-salt)	[81]

UMOD, uromodulin; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Figure Legends

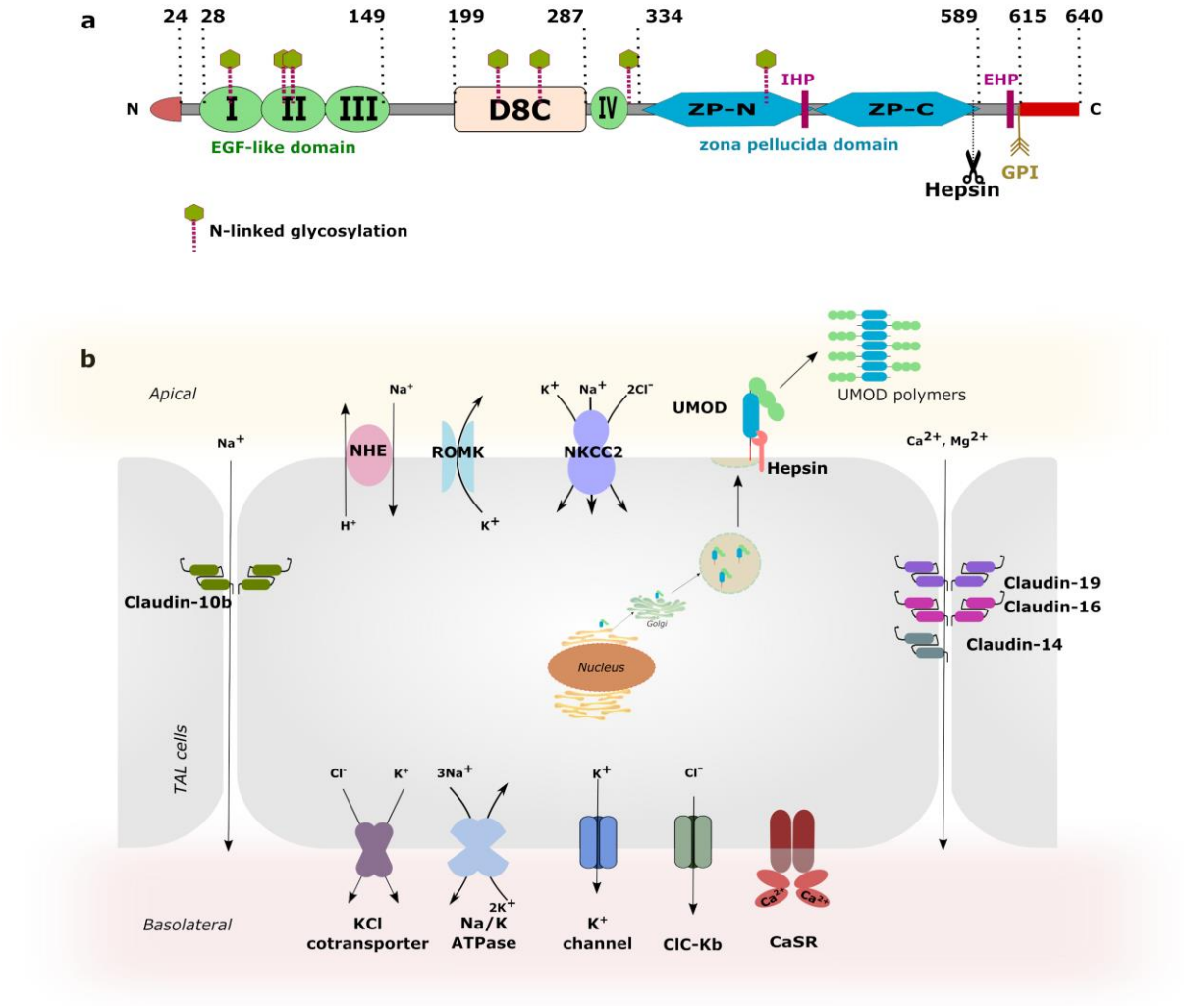
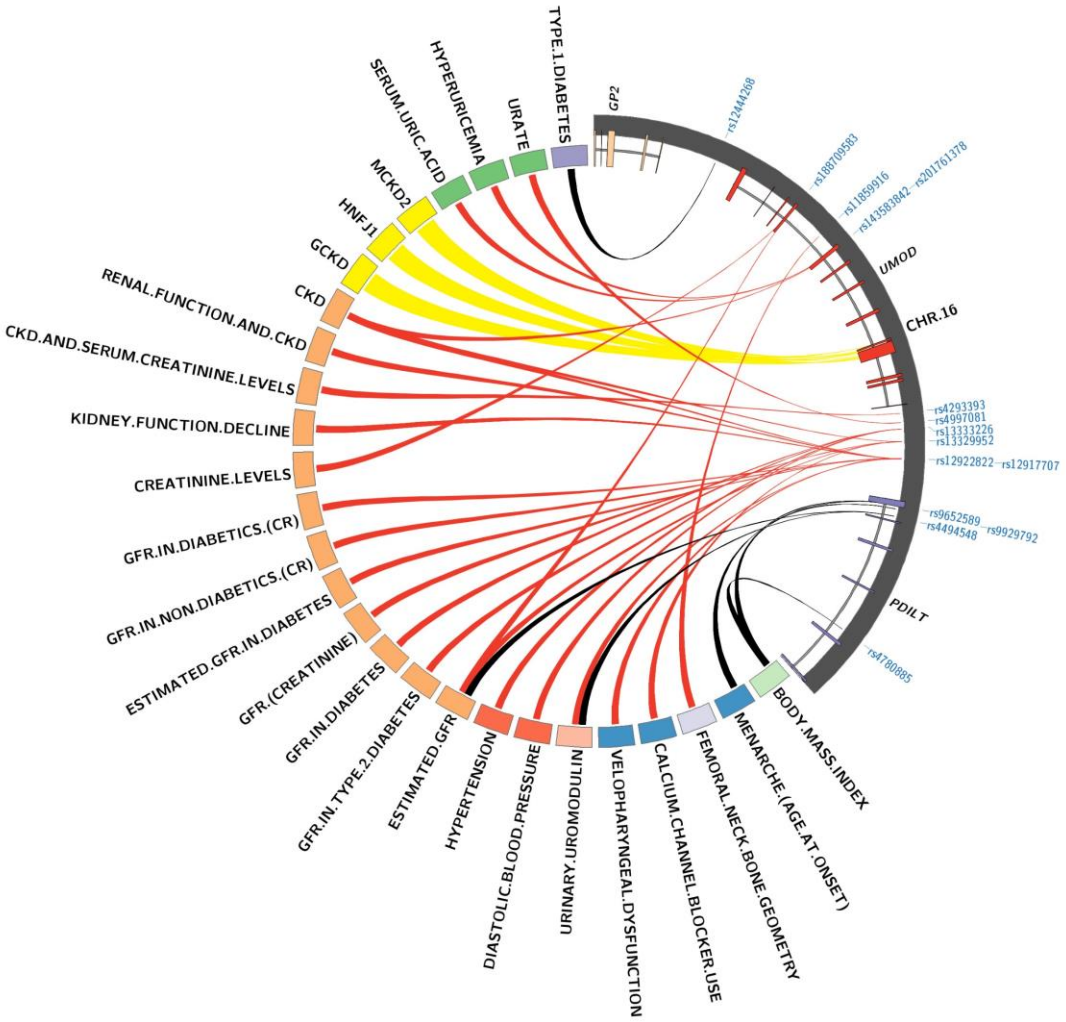


FIGURE 1. Uromodulin (UMOD) structure and thick ascending limb (TAL) physiology. **a**| The structure of UMOD contains four N-terminal epidermal growth factor (EGF)-like domains, a cysteine rich region (D8C), a C-terminal Zona Pellucida (ZP) module containing ZP-N and ZP-C domains, an internal hydrophobic patch (IHP) within the ZP linker region, an external hydrophobic patch (EHP), and a glycosylphosphatidylinositol (GPI) anchoring site. The hexagon shapes extending out from the structure represent the N-glycosylation sites. The serine protease hepsin cleaves UMOD at residues 586-589 at the C-terminal for release into the urine, leaving behind a membrane-attached peptide coloured in red. **b**| The TAL has

distinct basolateral (blood) and apical (urine) polarities and is involved in ion homeostasis. The apical transporters are Na^+/H^+ exchanger type 3 (NHE), renal outer medullary K^+ channel (ROMK), and Na^+-K^+-$2Cl^-$ cotransporter type 2 (NKCC2). The basolateral transporters include KCl cotransporter, Na^+-K^+-ATPase, K^+ channels, and the Chloride Channel Kb (CLC – Kb). The lumen-positive transepithelial potential generated by the combined action of these transporters drives the paracellular reabsorption of divalent cations via the Claudin 16/19 complex (in the outer stripe of the outer medulla) and Na^+ via Claudin-10b (in the inner stripe of the outer medulla). The arrows symbolise the direction of ion movement. Also depicted here is the secretory pathway of UMOD. UMOD is co-translationally inserted into the endoplasmic reticulum (ER), before extensive modification of glycan changes in the Golgi, and final cleavage of the polymerisation-incompetent form of UMOD by hepsin. The UMOD monomer is released into the urine in a polymerisation-competent form, where it assembles into macromolecular polymers. UMOD is also secreted via the basolateral membrane, in the form of serum UMOD. CaSR, calcium-sensing receptor.



1107

1108 **FIGURE 2.** Summary of all the phenotypic associations in and around the UMOD genomic
1109 locus. All significant SNPs identified by genome wide association studies with P<10-6 were
1110 obtained from GWAScatalog (PMID: 30445434) and monogenic syndromes from OMIM
1111 (https://omim.org/). ADTKD-UMOD, Autosomal dominant tubulointerstitial kidney disease
1112 caused by *UMOD* mutations; CKD, chronic kidney disease; GFR, glomerular filtration rate;
1113 *PDILT* gene (Protein Disulfide Isomerase Like, Testis Expressed); *GP2* gene (Glycoprotein
1114 2); CHR, chromosome.

1115

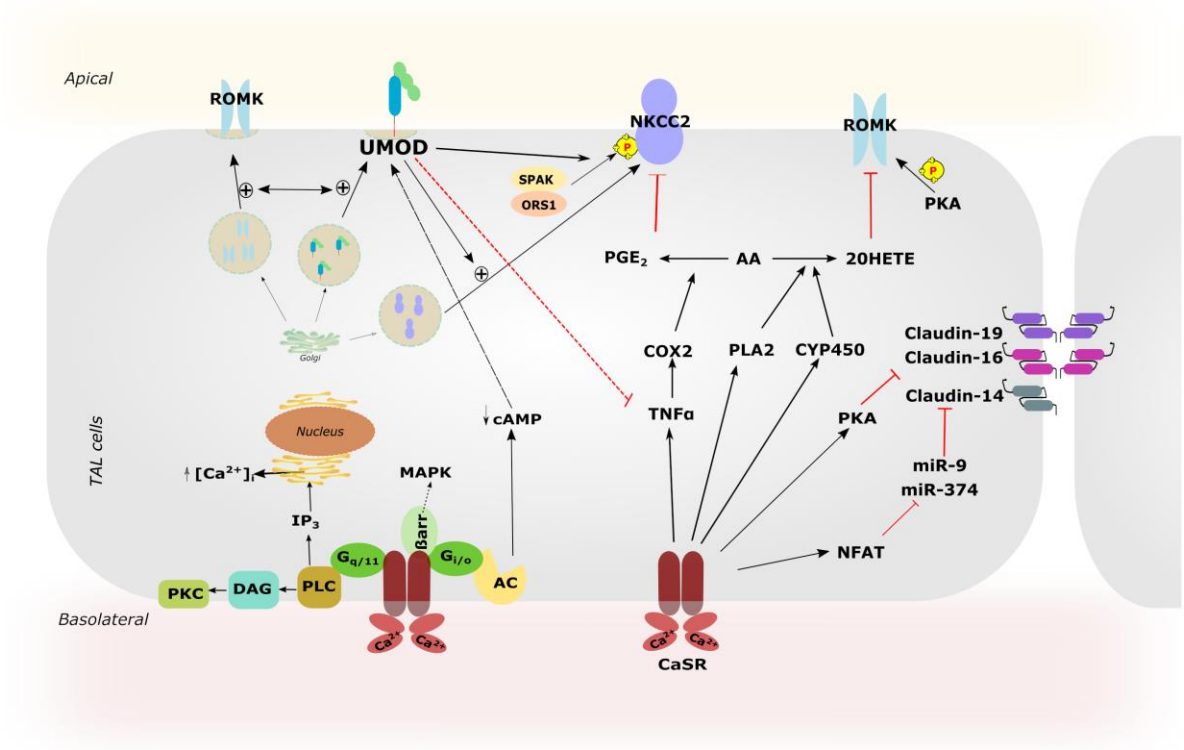


FIGURE 3. Signalling and regulation of various components of the thick ascending limb (TAL). Black arrows indicate stimulation and red T-lines indicate inhibition. Dashed lines indicate that the complete signalling cascade is unknown and are thus a prediction. Circular structures with a dashed circumference represent vesicles and the associated black arrows indicate exocytosis. The yellow circles with a red "P" in the centre indicate phosphorylation. AA, arachidonic acid; AC, adenylate cyclase; βarr, β-arrestin; cAMP, cyclic adenosine monophosphate; CaSR, calcium-sensing receptor; COX2, cyclooxygenase-2; CYP450, cytochrome P450; DAG, diacylglycerol; 20-HETE, 20-Hydroxyeicosatetraenoic acid; IP_3, inositol triphosphate; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells (NFAT); miR, microRNA; NKCC2, Na^+-K^+-$2Cl^-$ cotransporter type 2; OSR1, oxidative stress response 1 kinase; PGE_2, prostaglandin E_2; PKA, protein kinase A;

PKC, protein kinase C (PKC); PLA_2, phospholipase A_2; PLC, phospholipase C; ROMK, renal outer medullary K^+ channel; SPAK, SPS1-related proline-alanine-rich kinase; TNFα, tumour-necrosis factor alpha; UMOD, uromodulin.