GOZHENKO, Anatoliy, ZUKOW, Walery, GOZHENKO, Olena and IVANOV, Dmytro. The Extracellular Matrix of the Renal Medulla as a Dynamic Reservoir of Osmolytes. Pedagogy and Psychology of Sport. 2025;27:67029. elSSN 2450-6605.
https://doi.org/10.12775/PPS.2025.27.67029
https://apcz.umk.pl/PPS/article/view/67029

The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 29.10.2025. Revised: 20.11.2025. Accepted: 22.11.2025. Published: 01.12.2025.

The Extracellular Matrix of the Renal Medulla as a Dynamic Reservoir of Osmolytes

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ABSTRACT

The ability of mammalian kidneys to concentrate urine to 1200-1400 mOsm/kg is traditionally explained by the classical countercurrent multiplication theory; however, this model leaves at least three fundamental questions unanswered. First, the medullary osmotic gradient maintains remarkable stability even under high blood flow in the vasa recta, which theoretically should lead to massive washout of osmolytes; mathematical models demonstrate that countercurrent exchange alone cannot explain this resilience. Second, experimental studies from the 1960s-1980s revealed pleiotropic effects of antidiuretic hormone that extend far beyond simple regulation of aquaporin-2 at the epithelial level. Third, phenotypic variability in patients with similar V2 receptor or AQP2 mutations suggests the existence of additional modulating mechanisms whose nature remains enigmatic.

In this review, we integrate classical theory with contemporary data on the role of the medullary interstitial matrix, particularly hyaluronic acid, as a dynamic reservoir of osmolytes. Literature analysis confirms that HA concentration in the medulla is 3-5 times higher than in the cortex, and that this polyanionic macromolecule can immobilize Na^+ and urea through electrostatic interactions. Particularly compelling are the classical experiments of Rowen and Law (1981), which demonstrated that immunoneutralization of renal hyaluronidase with specific antiserum suppresses the concentrating response to ADH by 60-70%, leaving approximately one-third of the effect intact. This observation elegantly aligns with a dual-action model of ADH: the epithelial mechanism via V2R→cAMP→PKA→AQP2 provides rapid increase in water permeability, while the matrix mechanism via cAMP→hyaluronidase→HA depolymerization releases immobilized osmolytes, increasing the effective interstitial osmolarity. This multilevel integration not only explains the stability of the osmotic gradient under variable hemodynamic conditions but also opens new horizons for understanding the pathophysiology of urine concentrating defects and developing personalized therapeutic strategies.

Keywords: hyaluronic acid, extracellular matrix, antidiuretic hormone, aquaporins, urine concentration, renal medulla, countercurrent multiplication, osmoregulatory function of the kidneys

INTRODUCTION

When Werner Kuhn and Karl Ryffel first proposed the concept of countercurrent multiplication in 1942 to explain the ability of mammalian kidneys to produce hypertonic urine (Kuhn & Ryffel, 1942), they could hardly have anticipated how complex this system would prove to be upon detailed investigation. The experimental confirmation of their theory by Wirz and colleagues in 1951 (Wirz et al., 1951) seemed to put a period on this question, but subsequent decades of research revealed numerous aspects that the classical model could not satisfactorily explain. The discovery of aquaporins by Peter Agre in 1992 (Preston et al., 1992), for which he received the Nobel Prize in 2003 (Knepper & Nielsen, 2004), revolutionized our understanding of the molecular mechanisms of water transport; however, even this epochal discovery left some fundamental questions unanswered.

The central problem lies in the stability of the medullary osmotic gradient. The renal medulla receives approximately 5-10% of total renal blood flow through the vasa recta (Pallone et al., 2003), which represents a significant volume of fluid that could potentially wash out accumulated osmolytes. Classical theory states that the countercurrent organization of these vessels minimizes losses, as descending arterioles lose water and gain salts as they pass deeper into the medulla, while ascending venules perform reverse exchange (Pallone et al., 2000, 2003). However, mathematical modeling conducted by Layton and Layton (2005) convincingly showed that even an ideal countercurrent exchanger cannot completely prevent washout at realistic blood flow rates. This observation suggests the existence of additional stabilization mechanisms that have not yet been fully integrated into the general model of urine concentration (Sands & Layton, 2009).

A second intriguing aspect relates to the pleiotropic effects of antidiuretic hormone, which cannot be explained solely by regulation of aquaporin-2 (Brown, 2003; Nielsen et al., 1993, 1995). As early as 1962, Cobbin and Dicker made a curious observation: administration of vasopressin to rats correlated with the appearance of hyaluronidase activity in urine, with this activity peaking 2-4 hours after hormone injection (Cobbin & Dicker, 1962). At the time, this observation seemed curious but found no place in the dominant paradigm. Later, Law and Rowen (1981) demonstrated that antidiuretic stimulation leads to a 30-40% reduction in hexosamine content (components of glycosaminoglycans) in the rat renal medulla, with these changes being most pronounced in the inner medulla, where the osmotic gradient reaches maximum values. Most convincing, however, was the experiment by Rowen and Law that same year (Rowen & Law, 1981), when they used specific antiserum against renal hyaluronidase: immunoneutralization of this enzyme not only blocked the reduction in glycosaminoglycans but also suppressed the concentrating response to ADH by approximately two-thirds, leaving only 35-40% of the normal response. This elegant observation suggests that hyaluronidase plays not merely an auxiliary but a critical functional role in the mechanism of urine concentration (Stern & Jedrzejas, 2006).

The third question concerns phenotypic variability of clinical manifestations in genetic defects of the ADH-aquaporin system. Arthus and colleagues (2000) analyzed 117 families with X-linked nephrogenic diabetes insipidus and found striking heterogeneity: even with identical AVPR2 gene mutations, urine volume varied from 3 to 18 liters per day, and maximal urine osmolarity after desmopressin stimulation ranged from 80 to 350 mOsm/kg. If the defect is localized exclusively at the level of epithelial water transport (Robben et al., 2006), where does such variability come from? It is logical to assume the existence of additional modulating factors that can partially compensate for or, conversely, exacerbate the consequences of the primary genetic defect (Bichet, 2006; Fujiwara & Bichet, 2005).

We propose a hypothesis that integrates these disparate observations into a unified conceptual framework. Its essence is that the interstitial matrix of the renal medulla, particularly hyaluronic acid, functions as a dynamic reservoir of osmolytes, capable of immobilizing Na^+ and urea through electrostatic interactions due to its polyanionic character (Comper & Laurent, 1978; Laurent, 1964; Maroudas, 1968). Antidiuretic hormone acts through a dual mechanism: the classical epithelial pathway provides rapid increase in water permeability through aquaporin-2 trafficking (Hoffert et al., 2006; Nielsen et al., 1995), while a parallel matrix pathway activates hyaluronidase via a cAMP-dependent cascade (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981; Stern & Jedrzejas, 2006), leading to depolymerization of high-molecular-weight HA and release of immobilized osmolytes. This model not only explains the stability of the osmotic gradient at high blood flow

(Layton & Layton, 2005; Pallone et al., 2003) but also reconciles with classical experiments from the 1960s-1980s (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981) and predicts the existence of individual differences in HA metabolism as a factor modulating phenotypic expression of genetic defects (Arthus et al., 2000).

It is important to emphasize that this hypothesis is based primarily on experimental data obtained several decades ago (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), when the arsenal of molecular methods was significantly more limited than today. Therefore, critical validation using modern technologies—genetic knockouts (Kortenoeven & Fenton, 2014), real-time imaging (Schuh et al., 2018), proteomics (Hoffert et al., 2006), and metabolomics—is absolutely necessary before these concepts can be confidently integrated into clinical practice. Nevertheless, even at this stage, the intellectual appeal of the model and its explanatory power justify serious reconsideration of the role of the extracellular matrix in renal physiology (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991).

AIMS OF THE WORK

The aim of this review is a comprehensive analysis of the role of the interstitial matrix of the renal medulla, particularly hyaluronic acid (HA), in the mechanism of urine concentration (Comper & Laurent, 1978; Laurent & Fraser, 1992; Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006), integration of classical and contemporary concepts with emphasis on the dual action of ADH (epithelial and matrix) (Cobbin & Dicker, 1962; Hoffert et al., 2006; Law & Rowen, 1981; Nielsen et al., 1995; Rowen & Law, 1981), as well as identification of clinical implications (Arthus et al., 2000; Bichet, 2006; Eddy, 1996; Garantziotis & Savani, 2019) and directions for future research.

RESEARCH PROBLEMS

Problem 1: Classical countercurrent multiplication theory (Kuhn & Ryffel, 1942; Wirz et al., 1951) does not fully explain the stability of the medullary osmotic gradient at high blood flow in the vasa recta (up to 10% of renal blood flow) and variable hemodynamic conditions (Layton & Layton, 2005; Pallone et al., 2003; Sands & Layton, 2009).

Problem 2: Pleiotropic effects of ADH, documented in experimental studies from the 1960s-1980s (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), particularly changes in GAG content in the medulla, cannot be fully explained solely by regulation of AQP2 at the epithelial level (Brown, 2003; Nielsen et al., 1995).

Problem 3: The mechanism of immobilization of osmolytes (Na^+, urea) in the medullary interstitium and their controlled release under the influence of ADH remains insufficiently clarified at the molecular and biophysical levels, despite data on the electrostatic properties of HA (Comper & Laurent, 1978; Laurent, 1964; Maroudas, 1968).

Problem 4: Phenotypic variability of clinical manifestations in patients with similar mutations of V2R or AQP2 genes (diabetes insipidus) (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005) cannot be explained solely by defects in epithelial water transport (Robben et al., 2006), suggesting the role of additional modulating factors.

Problem 5: There are no validated biomarkers of renal medullary interstitial matrix function that could be used for diagnosis, prognosis, and monitoring of renal concentrating function disorders in clinical practice (Cowman et al., 2015; Garantziotis & Savani, 2019; Tammi et al., 2002).

RESEARCH HYPOTHESES

Hypothesis 1 (Reservoir Function of HA): Hyaluronic acid of the renal medullary interstitial matrix (concentration 50-100 μg/g, inner medulla/cortex gradient = 3-5:1) (Law & Rowen, 1981; Zhai et al., 2006) functions as a dynamic reservoir of osmolytes, immobilizing Na^+ and urea (20-40% of total amount under basal conditions) through electrostatic interactions (polyanionic character of HA, -1 charge per disaccharide) (Maroudas, 1968) and volume exclusion effect (high-molecular-weight HA $>10^6$ Da creates steric constraints) (Comper & Laurent, 1978; Laurent, 1964). This creates a buffer reservoir that protects the osmotic gradient from washout by blood flow in the vasa recta (Pallone et al., 2003), ensuring stability under variable hemodynamic conditions (Layton & Layton, 2005).

Hypothesis 2 (Dual Action of ADH): ADH acts through a dual synergistic mechanism: (A) classical epithelial pathway (V2R → cAMP → PKA → AQP2 trafficking) (Hoffert et al., 2006; Nielsen et al., 1995; Yasui et al., 1997) and (B) matrix pathway (V2R → cAMP → hyaluronidase activation → HA depolymerization → release of immobilized osmolytes) (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981; Stern & Jedrzejas, 2006).

Hypothesis 3 (Critical Role of Hyaluronidase): Activity of medullary hyaluronidase, regulated by the cAMP-dependent pathway (Cobbin & Dicker, 1962; Rowen & Law, 1981; Stern & Jedrzejas, 2006), is a critical

factor in modulating interstitial matrix properties (Comper & Laurent, 1978; Law & Rowen, 1981) and renal concentrating function (Rowen & Law, 1981; Sands & Layton, 2009).

Hypothesis 4 (Phenotypic Variability): Individual differences in HA metabolism (Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent & Fraser, 1992) determine phenotypic variability of renal concentrating capacity independently of AQP2 status (Arthus et al., 2000; Bichet, 2006; Robben et al., 2006).

Hypothesis 5 (Biomarkers): HA fragments of different molecular masses in urine and serum can serve as valid biomarkers of medullary interstitial matrix function (Cowman et al., 2015; Garantziotis & Savani, 2019; Tammi et al., 2002).

METHODS

This systematic narrative review was conducted in accordance with PRISMA recommendations for narrative syntheses (Page et al., 2021) and assessed using the SANRA scale (Baethge et al., 2019) to ensure methodological rigor. We performed a comprehensive literature search in five major electronic databases—PubMed/MEDLINE, Scopus, Web of Science Core Collection, Embase, and Cochrane Library—covering the period from January 1, 1942 (year of publication of Kuhn and Ryffel's pioneering work) to September 30, 2025. The search strategy was built around three main thematic blocks combined using Boolean operators: the first block included terms related to kidney structure and function ("kidney medulla", "loop of Henle", "collecting duct", "vasa recta", "countercurrent multiplication", "osmotic gradient"); the second block covered hormonal regulation ("antidiuretic hormone", "vasopressin", "V2 receptor", "aquaporin", "AQP2", "cAMP", "protein kinase A"); the third block concerned the extracellular matrix ("hyaluronic acid", "hyaluronan", "glycosaminoglycan", "extracellular matrix", "hyaluronidase", "hyaluronan synthase"). Additionally, we included clinical terms ("diabetes insipidus", "chronic kidney disease", "desmopressin") to identify relevant translational studies.

The primary search identified 1,847 potentially relevant publications, which after removal of duplicates using EndNote X9 software were reduced to 1,234 unique records. Two independent reviewers conducted screening by titles and abstracts, applying predefined inclusion and exclusion criteria; discrepancies that arose in 28 cases were resolved by consensus with participation of a third reviewer. Inclusion criteria encompassed original experimental and clinical studies, systematic reviews and meta-analyses, classical works in renal physiology, as well as publications in English, Ukrainian, Russian, and German in peer-reviewed journals. Conference abstracts without full text, editorial comments, publications of low methodological quality, and data duplicates were excluded. After screening, 312 publications were selected for full-text analysis.

Methodological quality assessment was conducted using validated tools specific to study type: SYRCLE's Risk of Bias tool for experimental animal studies (Hooijmans et al., 2014), Cochrane Risk of Bias tool 2.0 for clinical trials (Sterne et al., 2019), and AMSTAR 2 for systematic reviews (Shea et al., 2017). After detailed analysis, 156 publications were excluded due to insufficient relevance (89), low methodological quality (34), unavailability of full text (18), or data duplication (15), resulting in a final sample of 156 publications for systematic analysis. We paid special attention to classical experimental works from the 1960s-1980s, particularly studies by Cobbin and Dicker (1962), Law and Rowen (1981), and Rowen and Law (1981), which were identified through library archives, interlibrary loan, and digital repositories, as these pioneering works laid the foundation for understanding the interaction between antidiuretic hormone and hyaluronic acid metabolism.

The quality of evidence for each key statement was assessed using the GRADE system (Guyatt et al., 2011), which considers study design, risk of bias, inconsistency of results, indirect evidence, and imprecision of estimates. Classical countercurrent multiplication theory (Kuhn & Ryffel, 1942; Wirz et al., 1951) and the epithelial mechanism of ADH action through aquaporin-2 (Hoffert et al., 2006; Nielsen et al., 1995; Preston et al., 1992) received high quality ratings (⊕⊕⊕⊕), as they are confirmed by numerous independent studies using different methodological approaches. In contrast, the matrix mechanism of ADH action through hyaluronidase activation (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981) received a low rating (⊕⊕○○), mainly because most evidence comes from studies conducted several decades ago using methods now considered limited; contemporary validation using molecular and imaging technologies is critically needed. Clinical relevance of HA fragment-based biomarkers (Cowman et al., 2015; Garantziotis & Savani, 2019) received a very low rating (⊕○○○), as available data are predominantly preliminary and require confirmation in large prospective cohorts.

In accordance with recommendations of the International Committee of Medical Journal Editors (ICMJE) and the Committee on Publication Ethics (COPE) regarding transparency in the use of artificial intelligence tools, we declare that the AI assistant Claude (Anthropic, Claude 3.5 Sonnet model) was used exclusively for technical assistance in text structuring, grammar checking, reference formatting, and optimization of database search queries. No AI-generated text was included in the manuscript without critical analysis, editing, and verification by the authors; all scientific concepts, hypotheses, data interpretations, and conclusions are the original intellectual contribution of the authors, who bear full responsibility for the accuracy and scientific integrity of the work. AI use was limited to auxiliary functions and did not replace critical thinking, scientific expertise, or creative contribution of the researchers.

Due to the heterogeneity of included studies—different experimental models, methodological approaches, and endpoints—quantitative meta-analysis was not feasible, so we conducted a narrative synthesis with thematic organization of material. Limitations of our methodological approach include possible omission of relevant publications in other languages, potential publication bias favoring positive results, limited number of contemporary studies of the matrix mechanism, and the inherent tendency of narrative reviews toward subjective interpretation. Despite these limitations, the systematic nature of the search, use of validated quality assessment tools (Baethge et al., 2019; Guyatt et al., 2011; Hooijmans et al., 2014; Page et al., 2021; Shea et al., 2017; Sterne et al., 2019), and involvement of experts from different disciplines ensure sufficient rigor for formulating well-founded conclusions and hypotheses.

CLASSICAL THEORY: COUNTERCURRENT MULTIPLICATION AND ITS LIMITS

The architecture of the loop of Henle represents a brilliant evolutionary solution to the problem of urine concentration, based on spatial separation of ion and water transport in different nephron segments (Sands & Layton, 2009). The thick ascending limb of the loop of Henle functions as the "engine" of the entire system: here active NaCl transport occurs through the apical cotransporter NKCC2, which transports one sodium ion, one potassium ion, and two chloride ions into the cell, using the energy of the sodium electrochemical gradient created by the basolateral Na^+-K^+-ATPase (Hebert et al., 2004). Potassium recycles back into the lumen through apical ROMK (Kir1.1) channels, maintaining substrate for the cotransporter, while chloride exits the cell through basolateral ClC-Kb channels in complex with the regulatory subunit barttin (Estévez et al., 2001); mutations of any of these components lead to Bartter syndrome with characteristic salt wasting and inability to concentrate urine (Estévez et al., 2001; Hebert et al., 2004). A critically important feature of the thick ascending limb is the absence of aquaporins, making this segment virtually impermeable to water: fluid passing through it becomes progressively hypotonic (to 100-150 mOsm/kg), while the medullary interstitium becomes enriched with reabsorbed salt, creating an osmotic gradient (Sands & Layton, 2009).

The thin descending limb of the loop of Henle represents the opposite of the thick ascending limb: here aquaporin-1 expression reaches extremely high levels (over ten million molecules per cell), providing exceptional water permeability (Agre et al., 1993; Fushimi et al., 1993; Pallone et al., 2000). When hypotonic fluid from the proximal tubule enters this limb and descends into the medulla, it encounters progressively increasing interstitial osmolarity; water rapidly exits the lumen down the osmotic gradient, concentrating the fluid inside the tubule. At the deepest point of the loop, in the inner medulla, tubular fluid osmolarity can reach 1200-1400 mOsm/kg, almost completely equilibrating with the surrounding interstitium (Sands & Layton, 2009). The thin ascending limb, found only in the inner medulla in long-looped nephrons, demonstrates yet another unique property: it is permeable to NaCl but not to water, allowing passive salt reabsorption down the concentration gradient without accompanying water movement (Sands & Layton, 2009). This elegant organization creates a "multiplication effect": a small single osmotic gradient between ascending and descending limbs at each horizontal level (approximately 200 mOsm/kg) is multiplied along the vertical axis, creating a cumulative gradient 4-5 times larger (Kuhn & Ryffel, 1942; Wirz et al., 1951).

The collecting duct serves as the final control point, where the organism makes the decision about final urine osmolarity depending on water balance needs (Brown, 2003; Nielsen et al., 1995). Without antidiuretic hormone, principal cells of the collecting duct are characterized by extremely low apical water permeability: most aquaporin-2 molecules are sequestered in intracellular vesicles, with only a small fraction present on the apical membrane (Nielsen et al., 1993, 1995). Fluid entering from the distal convoluted tubule (already hypotonic after passing through the thick ascending limb) passes through the collecting duct with virtually no water reabsorption, despite the enormous osmotic gradient between lumen (100-150 mOsm/kg) and medullary interstitium (1200-1400 mOsm/kg). The result is a large volume of hypotonic urine—a state we call water diuresis (Sands & Layton, 2009). Binding of antidiuretic hormone to the V2 receptor on the basolateral membrane of principal cells initiates a cascade of events: activation of adenylyl cyclase through Gs protein, elevation of intracellular cAMP, activation of protein kinase A, phosphorylation of aquaporin-2 at critical serine residues (especially Ser256) (Hoffert et al., 2006), and trafficking of vesicles to the apical membrane (Nielsen et al., 1995). Within 15-30 minutes, the number of functional AQP2 molecules on the apical surface increases 5-10 fold, water permeability dramatically increases, and fluid begins to equilibrate with the hypertonic interstitium, producing concentrated urine (Brown, 2003; Nielsen et al., 1995).

However, even this elegant system faces a fundamental problem: the medulla is not an isolated compartment; it is constantly perfused with blood through the vasa recta, which must deliver oxygen and nutrients as well as remove metabolic waste (Pallone et al., 2003). Classical theory states that the countercurrent organization of these vessels—descending arterioles and ascending venules running parallel to each other—minimizes washout of the osmotic gradient (Pallone et al., 2000, 2003). As blood descends into the medulla through descending arterioles, it loses water (which exits down the osmotic gradient) and gains NaCl and urea (which enter down concentration gradients), progressively equilibrating with the surrounding interstitium; at the deepest point, plasma osmolarity can reach 1200 mOsm/kg (Pallone et al., 2003). During ascent through ascending

venules, the reverse process occurs: water is reabsorbed back into the vascular bed, and solutes diffuse outward, so that by the time of exit from the medulla, plasma returns to an iso-osmotic state (Pallone et al., 2003). At first glance, this is an ideal solution, but mathematical modeling revealed a critical problem.

Layton and Layton (2005) in their influential work created a detailed computer model of the concentrating mechanism that accounted for actual nephron geometry, transporter properties, membrane permeabilities, and vasa recta hemodynamics. Their results were unexpected: even assuming ideal countercurrent exchange (100% equilibration efficiency between descending and ascending vessels) and at realistic blood flow rates (5-10% of total renal blood flow, approximately 50-100 ml/min for both kidneys in humans), the model predicted significantly more osmolyte washout than is observed in vivo. In other words, the experimentally measured osmotic gradient proved more stable than theoretically possible solely through countercurrent exchange (Layton & Layton, 2005). This discrepancy between theory and experiment is not a trivial error—it amounts to approximately 20-30% of the gradient magnitude, which is too much to ignore. The authors suggested the existence of additional stabilization mechanisms, possibly related to interstitial space properties (Layton & Layton, 2005), but at that time lacked sufficient data to specify this hypothesis.

Another observation difficult to reconcile with the classical model concerns dynamic response to hemodynamic changes. During physical exercise or postural changes, blood flow in the vasa recta can fluctuate widely, yet renal concentrating capacity remains remarkably stable in healthy individuals (Pallone et al., 2003; Sands & Layton, 2009). If the osmotic gradient is maintained exclusively by the balance between active transport in the thick ascending limb and washout through the vasa recta, we would expect much greater variability (Layton & Layton, 2005). This suggests the existence of a "buffering system"—a reservoir of osmolytes that can temporarily immobilize Na^+ and urea, protecting them from washout by blood flow, and release them as needed. The nature of this hypothetical reservoir remained a mystery for a long time, but accumulated data point to the medullary interstitial matrix, particularly hyaluronic acid, as the most likely candidate for this role (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006).

MOLECULAR REVOLUTION: AQUAPORINS AND SIGNALING CASCADES

The discovery of the first water channel by Preston, Carroll, Guggino, and Agre in 1992 (Preston et al., 1992) was a true breakthrough in understanding cellular water transport, for which Peter Agre was awarded the Nobel Prize in Chemistry in 2003 together with Roderick MacKinnon, who deciphered the structure of ion channels (Knepper & Nielsen, 2004). Until that moment, the mechanism by which water crosses lipid membranes at rates exceeding simple diffusion by thousands of times remained one of the greatest mysteries of cellular physiology (Agre et al., 1993). Agre and colleagues identified a 28-kilodalton protein in erythrocyte membranes, which they initially called CHIP28 (channel-forming integral protein of 28 kDa), and later renamed aquaporin-1 (Preston et al., 1992); expression of this protein in Xenopus oocytes conferred extraordinary water permeability that could be blocked by mercury ions (Preston et al., 1992). Subsequent years brought a cascade of discoveries: at least thirteen members of the aquaporin family were identified in mammals, each with specific tissue localization and regulatory properties (Agre et al., 1993; Kortenoeven & Fenton, 2014).

In the kidneys, at least eight different aquaporins are expressed, but three of them have particular significance for urine concentration (Agre et al., 1993; Kortenoeven & Fenton, 2014). Aquaporin-1 is constitutively expressed at high concentrations in the proximal tubule, descending limb of the loop of Henle, and vasa recta endothelium (Fushimi et al., 1993; Pallone et al., 2000); its function is not hormonally regulated, and it provides baseline high water permeability in these segments. Deletion of the AQP1 gene in mice leads to a moderate urine concentrating defect (maximal osmolarity decreases by approximately 40%), but animals remain viable, suggesting the existence of compensatory mechanisms (Pallone et al., 2000). Aquaporin-2 is the star of our story: this protein is expressed exclusively in principal cells of the collecting duct and is the only family member that undergoes rapid hormonal regulation through trafficking between intracellular vesicles and the apical membrane (Brown, 2003; Nielsen et al., 1993, 1995). Nielsen and colleagues in 1995 (Nielsen et al., 1995) elegantly demonstrated using immunofluorescence microscopy that in the absence of vasopressin, AQP2 is localized predominantly in subapical vesicles, whereas hormone stimulation leads to its massive translocation to the apical surface within 15-30 minutes. Aquaporins-3 and -4 are localized on the basolateral membrane of principal cells and provide water exit from the cell into the interstitium; their expression is also regulated by vasopressin, but at the transcriptional level during chronic stimulation (Kortenoeven & Fenton, 2014; Yasui et al., 1997).

The molecular cascade connecting vasopressin binding to the cell surface receptor with the appearance of water channels on the apical membrane was deciphered through the efforts of many laboratories during the 1990s-2000s (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995; Robben et al., 2006). The V2 receptor belongs to the G protein-coupled receptor family and has the classic structure with seven transmembrane domains; binding of arginine-vasopressin to extracellular loops of the receptor induces a conformational change that activates the associated Gs protein on the intracellular side of the membrane (Robben et al., 2006). The activated αs subunit of Gs dissociates from the $\beta\gamma$ complex and binds to adenylyl cyclase type 6 (the main isoform in the collecting duct),

stimulating synthesis of cyclic adenosine monophosphate from ATP (Robben et al., 2006). The concentration of cAMP in the cytoplasm rapidly increases from a basal level of approximately 5 nanomolar to 50-100 nanomolar within a few minutes; this universal second messenger molecule binds to regulatory subunits of protein kinase A, causing their dissociation and release of catalytic subunits (Hoffert et al., 2006; Robben et al., 2006).

Active protein kinase A phosphorylates multiple substrates, but for our story, the most critical is phosphorylation of the C-terminal tail of aquaporin-2 (Hoffert et al., 2006). Hoffert and colleagues in 2006 (Hoffert et al., 2006) conducted an elegant proteomic study using mass spectrometry to identify all AQP2 phosphorylation sites in response to vasopressin; they found that at least four serine residues (Ser256, Ser264, Ser269, and Ser261) can be phosphorylated, with Ser256 being most critical for trafficking. Mutation of this residue to alanine (which cannot be phosphorylated) virtually completely blocks AQP2 translocation to the apical membrane in response to vasopressin, whereas mutations of other sites have less dramatic effects (Hoffert et al., 2006). Phosphorylation of Ser256 creates a recognition signal for trafficking machinery proteins: molecular motors (dynein, kinesin) transport vesicles along microtubules and actin filaments to the apical membrane, where SNARE proteins (syntaxin-4, VAMP2, SNAP-23) mediate vesicle fusion with the plasma membrane (Brown, 2003; Nielsen et al., 1995). The entire process from hormone binding to appearance of functional water channels on the cell surface takes approximately 15-30 minutes, consistent with clinical observations of desmopressin antidiuretic effect onset (Brown, 2003; Nielsen et al., 1995).

However, the story does not end there, as chronic vasopressin stimulation (over 24 hours) induces an additional level of regulation at the transcriptional level (Yasui et al., 1997). Yasui and colleagues in 1997 (Yasui et al., 1997) showed that the AQP2 gene promoter contains cAMP-response elements (CRE)—specific DNA sequences that bind the transcription factor CREB (cAMP response element-binding protein) after its phosphorylation by protein kinase A. Activation of this pathway leads to increased AQP2 gene transcription, 2-3 fold increase in mRNA levels, and 3-5 fold accumulation of AQP2 protein within 24-48 hours (Yasui et al., 1997). This adaptive mechanism allows the kidney to maintain high concentrating capacity during chronic states requiring prolonged antidiuresis, such as heart failure or liver cirrhosis (Kortenoeven & Fenton, 2014; Yasui et al., 1997). Interestingly, the opposite situation—chronic water deprivation or primary polydipsia with chronic suppression of vasopressin secretion—leads to decreased AQP2 expression, a phenomenon known as "escape" or "washout," which explains why patients with compulsive water drinking gradually lose the ability to concentrate urine even after cessation of excessive drinking (Kortenoeven & Fenton, 2014).

The discovery of aquaporins and deciphering of the vasopressin signaling cascade is undoubtedly a triumph of molecular biology (Agre et al., 1993; Hoffert et al., 2006; Knepper & Nielsen, 2004; Preston et al., 1992), but it also revealed new mysteries. Why, for example, do patients with identical AVPR2 gene mutations (encoding the V2 receptor) show such significant phenotypic variability? Arthus and colleagues (2000) analyzed 117 families and found that even brothers with the same mutation can have urine volumes differing 2-3 fold and maximal osmolarity after desmopressin stimulation varying from 80 to 350 mOsm/kg. If the defect is localized at the receptor level and the entire downstream cascade (adenylyl cyclase, PKA, AQP2) is intact (Robben et al., 2006), where does such variability come from? The standard explanation refers to "modifier genes" or "epigenetic factors," but these are more labels for our ignorance than true explanations (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005). We propose an alternative hypothesis: individual differences in medullary interstitial matrix metabolism, particularly in the activity of hyaluronic acid synthases and hyaluronidases (Cowman et al., 2015; Garantziotis & Savani, 2019; Stern & Jedrzejas, 2006), may modulate urine concentrating efficiency independently of epithelial water transport status, creating an additional level of variability not previously accounted for in clinical studies (Arthus et al., 2000).

HYALURONIC ACID: THE FORGOTTEN PLAYER IN RENAL PHYSIOLOGY

Hyaluronic acid, also known as hyaluronan, is the simplest in structure but one of the most interesting in function among the glycosaminoglycan family (Comper & Laurent, 1978; Laurent & Fraser, 1992). Unlike other GAGs such as chondroitin sulfate or heparan sulfate, HA does not contain sulfate groups and does not form covalent bonds with a protein core, i.e., technically is not a proteoglycan but exists as a free polysaccharide (Laurent & Fraser, 1992). Its molecular structure consists of repeating disaccharide units, each containing D-glucuronic acid connected by a β(1→3)-glycosidic bond to N-acetyl-D-glucosamine, which in turn is connected by a β(1→4) bond to the next glucuronic acid; this simple repeating structure can polymerize to extraordinary sizes (Comper & Laurent, 1978; Laurent & Fraser, 1992). The molecular mass of HA in tissues varies from several thousand daltons (oligosaccharides containing 5-10 disaccharide units) to over ten million daltons (high-molecular-weight HA containing more than 25,000 disaccharides and reaching lengths of 10 micrometers, comparable to the diameter of an entire cell) (Cowman et al., 2015; Laurent & Fraser, 1992).

The physicochemical properties of hyaluronic acid are determined by its polyanionic character: each disaccharide unit contains one carboxyl group of D-glucuronic acid with a pKa of approximately 3, meaning complete ionization at physiological pH 7.4 and creation of high negative charge density along the polymer chain (one negative charge per 400 daltons of molecular mass) (Maroudas, 1968). This negative charge has profound

consequences for HA interaction with the surrounding environment (Comper & Laurent, 1978; Laurent, 1964; Maroudas, 1968). First, negative charges repel each other, forcing the molecule to adopt an extended conformation in solution; the radius of gyration of an HA molecule with molecular mass of one million daltons is approximately 200-300 nanometers, tens of times larger than typical globular proteins of similar mass (Comper & Laurent, 1978). Second, negative charges attract counterions (predominantly Na^+ under physiological conditions), creating an "ionic atmosphere" around the polymer; Donnan theory predicts that local Na^+ concentration in immediate proximity to HA can be 1.5-2 times higher than in free solution, effectively immobilizing a portion of cations (Maroudas, 1968).

Third, and perhaps most important for our story, HA demonstrates extraordinary hygroscopic properties: one molecule can bind from one to ten thousand water molecules, depending on molecular mass and concentration (Comper & Laurent, 1978; Laurent & Fraser, 1992). The mechanisms of this binding are multiple: hydrogen bonds between hydroxyl and carboxyl groups of HA and H_2O molecules; osmotic pressure created by negative charges and associated counterions that attracts water; formation of branched hydration domains where water molecules are organized in structured layers around the polymer (Comper & Laurent, 1978). Comper and Laurent in their classic 1978 work (Comper & Laurent, 1978) showed that HA solutions even at relatively low concentrations (1-2 mg/ml) demonstrate gel properties with high viscosity and elasticity, creating a three-dimensional network that fills space and restricts movement of other macromolecules. This phenomenon, known as the "volume exclusion effect," is critical for interstitial space organization: large HA molecules create steric barriers that affect diffusion of proteins, lipoproteins, and even small molecules, effectively compartmentalizing the interstitium at the microscale (Comper & Laurent, 1978; Laurent, 1964).

The distribution of hyaluronic acid in the kidney is not uniform, and it is precisely this gradient that provides the first hints of its functional significance in urine concentration (Law & Rowen, 1981; Zhai et al., 2006). Zhai and colleagues in 2006 (Zhai et al., 2006) conducted a detailed immunohistochemical study using a biotinylated probe that specifically binds HA (bHABP, isolated from bovine brain); their results revealed a clear cortico-medullary gradient. In the renal cortex, staining was minimal, with HA concentration approximately 5-15 micrograms per gram of tissue, localized predominantly in perivascular zones around large vessels (Zhai et al., 2006). In the outer medulla, staining intensity moderately increased, with concentration of 20-40 micrograms per gram, with HA detected in the interstitium between tubules and vessels (Zhai et al., 2006). The most dramatic changes were observed in the inner medulla: here HA concentration reached 50-100 micrograms per gram (3-5 times higher than in cortex), with intense interstitial staining, especially around collecting ducts and thin limbs of loops of Henle (Zhai et al., 2006). This spatial distribution is not random—it almost perfectly correlates with the osmotic gradient: the osmolarity ratio between inner medulla and cortex is approximately 4-5:1 (1200-1400 mOsm/kg versus 290 mOsm/kg), remarkably close to the HA concentration ratio (3-5:1) (Law & Rowen, 1981; Sands & Layton, 2009; Zhai et al., 2006).

Biochemical analysis conducted by Law and Rowen in 1981 (Law & Rowen, 1981) on isolated rat kidney zones confirmed these immunohistochemical observations and added important details. They measured hexosamine content (components of all glycosaminoglycans) by the Elson-Morgan method after acid hydrolysis of tissue and found that in the inner medulla, total GAG content is 120-150 micrograms per gram, with approximately 60-70% of this amount attributable to hyaluronic acid (the remainder predominantly chondroitin sulfate and dermatan sulfate) (Law & Rowen, 1981). Gel filtration chromatography of extracts showed that under basal conditions (rats with free access to water), medullary HA is represented predominantly by high-molecular-weight forms with an elution peak corresponding to molecular mass of 800,000 - 1.5 million daltons; approximately 70-80% of total HA had molecular mass over one million daltons (Law & Rowen, 1981). This high-molecular-weight form is optimal for creating a viscous gel and immobilizing ions through electrostatic interactions (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968).

The source of hyaluronic acid in the medulla is specialized cells known as renal medullary interstitial cells (RMIC), first described in detail by Lemley and Kriz in 1991 (Lemley & Kriz, 1991) and Kaissling and Le Hir in 2008 (Kaissling & Le Hir, 2008). These fibroblast-like cells with long processes form a three-dimensional network in the interstitium between tubules and vessels, comprising approximately 5-10% of all medullary cells (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991). Morphologically they resemble myofibroblasts with a spindle-shaped body and numerous cytoplasmic processes that contact basement membranes of tubules and capillaries; their cytoplasm contains characteristic lipid droplets believed to be reservoirs of prostaglandin precursors (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991). RMIC markers include ecto-5'-nucleotidase (CD73), vimentin, and, upon activation or fibrosis, α-smooth muscle actin (Kaissling & Le Hir, 2008; Mutsaers et al., 2015). Functionally, these cells are extremely versatile: they synthesize extracellular matrix components (collagen types I and III, fibronectin, glycosaminoglycans), produce vasoactive prostaglandins (PGE_2 and PGI_2, which have protective vasodilatory effects in the medulla), respond to osmotic stress, and participate in tissue repair after injury (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991; Mutsaers et al., 2015).

Hyaluronic acid synthesis is carried out by a family of enzymes known as hyaluronan synthases (HAS), of which three isoforms have been identified in mammals: HAS1, HAS2, and HAS3 (Cowman et al., 2015;

Garantziotis & Savani, 2019; Tammi et al., 2002). All three are expressed in RMIC but with different intensities and regulation (Garantziotis & Savani, 2019). HAS2 is the main isoform, constitutively expressed at high levels and producing high-molecular-weight HA (over one million daltons); HAS2 expression is regulated by transcription factors sensitive to osmotic stress (TonEBP/NFAT5), growth factors (TGF-β, PDGF), and hypoxia (HIF-1α) (Garantziotis & Savani, 2019; Tammi et al., 2002). HAS1 has low basal expression but can be induced under pathological conditions, especially hypoxia (Garantziotis & Savani, 2019). HAS3 is moderately expressed and produces medium-molecular-weight HA (100-500 thousand daltons) (Cowman et al., 2015; Garantziotis & Savani, 2019). Interestingly, increased interstitial osmolarity, which occurs during water deprivation, stimulates HAS2 expression through TonEBP activation, creating negative feedback: water loss from the interstitium stimulates HA synthesis, which binds water and partially compensates for dehydration (Garantziotis & Savani, 2019).

Degradation of hyaluronic acid is carried out by a family of hyaluronidase enzymes, of which four have been identified in mammals: HYAL1, HYAL2, HYAL3, and HYAL4, although the functions of the last two remain insufficiently clarified (Stern & Jedrzejas, 2006). HYAL1 is a lysosomal endoglycosidase with pH optimum of 3.5-4.0 that degrades HA to tetrasaccharides and hexasaccharides; it is responsible for intracellular HA catabolism after endocytosis through CD44 or HARE receptors (Stern & Jedrzejas, 2006). HYAL2 is a membrane endoglycosidase anchored on the outer side of the plasma membrane through a GPI anchor, with pH optimum of 6.0-7.0; it cleaves high-molecular-weight HA to fragments of approximately 20 kilodaltons (approximately 50 disaccharide units), which can then be internalized and further degraded by HYAL1 (Stern & Jedrzejas, 2006). It is precisely HYAL2, due to its extracellular localization and neutral pH optimum, that is the most likely candidate for the role of the enzyme modulating medullary interstitial matrix properties in response to antidiuretic hormone (Cobbin & Dicker, 1962; Rowen & Law, 1981; Stern & Jedrzejas, 2006), although direct evidence of this assumption remains limited.

CLASSICAL EXPERIMENTS: FORGOTTEN EVIDENCE OF THE MATRIX MECHANISM

The history of science abounds with examples of observations that were ahead of their time and were forgotten or ignored because they did not fit the dominant paradigm; only decades later, when the conceptual framework changed, do these observations suddenly acquire new significance (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). Such was the fate of a series of elegant experiments conducted in the 1960s-1980s by a group of researchers studying the interaction between antidiuretic hormone and glycosaminoglycan metabolism in the kidneys. At that time, these works generated some interest but quickly moved to the periphery of scientific discourse, as the molecular mechanisms of vasopressin action were still unknown (aquaporins would be discovered only decades later by Preston et al., 1992), and methods for studying the extracellular matrix were limited. Now, armed with modern understanding of signaling cascades (Hoffert et al., 2006; Nielsen et al., 1995; Robben et al., 2006) and matrix structure (Comper & Laurent, 1978; Garantziotis & Savani, 2019; Laurent & Fraser, 1992), we can reassess these classical experiments and see in them convincing evidence of a mechanism that deserves serious attention.

The pioneering observation belongs to Cobbin and Dicker, who in 1962 (Cobbin & Dicker, 1962) published a short article in the Journal of Physiology titled "Antidiuretic activity of hyaluronidase." Their experiment was conceptually simple, but the results proved intriguing. Rats were administered vasopressin (100-500 milliunits intravenously or subcutaneously) and urine was collected over the next 6-8 hours; hyaluronidase activity in urine was measured by a turbidimetric method based on the enzyme's ability to reduce turbidity of hyaluronic acid solution (Cobbin & Dicker, 1962). In control animals, hyaluronidase activity in urine was minimal or undetectable, but after vasopressin administration it began to increase after 30-60 minutes, reached a maximum at 2-4 hours, and gradually decreased over the next 4-6 hours (Cobbin & Dicker, 1962). Importantly, activity correlated with hormone dose: low doses (100 milliunits) caused moderate elevation, while high doses (500 milliunits) led to a 5-7 fold increase in activity compared to baseline (Cobbin & Dicker, 1962). Cobbin and Dicker (1962) also conducted an interesting control experiment: they administered exogenous hyaluronidase (isolated from bull testes) together with a suboptimal dose of vasopressin and found a synergistic effect—the antidiuretic response was significantly stronger than with vasopressin alone. This observation suggested that hyaluronidase is not merely a byproduct of hormone action but may play a functional role in the urine concentrating mechanism (Cobbin & Dicker, 1962; Stern & Jedrzejas, 2006).

However, these early experiments had significant limitations (Cobbin & Dicker, 1962). First, the source of hyaluronidase in urine was not unambiguously determined: the enzyme could originate from the renal medulla (which would support the hypothesis of interstitial matrix modulation) but could also be released from other tissues or even from urinary tract epithelium. Second, the correlation between appearance of enzymatic activity and antidiuretic effect did not prove a causal relationship—both phenomena could be independent consequences of vasopressin action. Third, methods of that time did not allow identification of the specific hyaluronidase isoform (Stern & Jedrzejas, 2006) or determination of its precise localization in the kidney. Nevertheless, these pioneering

observations opened the door for more detailed studies that appeared almost two decades later (Law & Rowen, 1981; Rowen & Law, 1981).

Law and Rowen in 1981 (Law & Rowen, 1981) approached the problem from another angle: instead of measuring enzymatic activity, they directly assessed changes in glycosaminoglycan content in different kidney zones. Their experimental design was carefully thought out and included three groups of rats: a control group with free access to water, a group with water deprivation for 24 or 48 hours (physiological stimulation of endogenous vasopressin), and a group with infusion of exogenous vasopressin (5 milliunits per hour for 6 hours) with free access to water (Law & Rowen, 1981). After euthanasia, kidneys were rapidly removed, dissected along the cortico-medullary axis, and under microscope separated into three zones: cortex, outer medulla, and inner medulla (Law & Rowen, 1981). Each zone was weighed, homogenized, and glycosaminoglycans were extracted using papain (to cleave protein bonds) followed by ethanol precipitation; hexosamine content in the extract was determined colorimetrically by the Elson-Morgan method after acid hydrolysis (Law & Rowen, 1981).

The results were striking in their clarity (Law & Rowen, 1981). In control animals, hexosamine content in the inner medulla was 120±15 micrograms per gram of wet tissue (mean ± standard deviation), in the outer medulla 65±10 micrograms per gram, and in the cortex only 35±8 micrograms per gram, confirming the cortico-medullary gradient we discussed earlier (Law & Rowen, 1981; Zhai et al., 2006). After 24 hours of water deprivation, hexosamine content in the inner medulla dramatically decreased to 75±12 micrograms per gram (37% reduction, $p<0.001$), while in the outer medulla the decrease was more moderate—to 50±9 micrograms per gram (23%, $p<0.01$), and in the cortex practically unchanged (32±7 micrograms per gram, difference statistically insignificant) (Law & Rowen, 1981). Infusion of exogenous vasopressin for 6 hours caused similar, though somewhat less pronounced changes: hexosamine content in the inner medulla decreased to 70±10 micrograms per gram (42% reduction, $p<0.001$) (Law & Rowen, 1981). Importantly, these changes correlated with functional parameters: urine osmolarity increased from 580±80 mOsm/kg in controls to 1150±120 mOsm/kg after deprivation or vasopressin infusion, and urine volume decreased from 8.5±1.2 ml per 6 hours to 2.1±0.4 ml (Law & Rowen, 1981).

Gel filtration chromatography of extracts provided additional information about the nature of changes (Law & Rowen, 1981). In control animals, medullary glycosaminoglycans eluted predominantly in the high-molecular-weight fraction (void volume of Sepharose CL-4B column, corresponding to molecular mass over 1 million daltons), whereas after antidiuretic stimulation a significant fraction appeared in the medium-molecular-weight range (100-500 thousand daltons) and low-molecular-weight range (10-50 thousand daltons) (Law & Rowen, 1981). This indicated not merely a reduction in total GAG amount but their depolymerization—exactly what is expected with hyaluronidase activation (Cobbin & Dicker, 1962; Law & Rowen, 1981; Stern & Jedrzejas, 2006). Treatment of extracts with specific enzymes (chondroitinase ABC for degradation of sulfated GAGs, hyaluronidase for HA degradation) showed that approximately 60-70% of hexosamines in the medulla originate from hyaluronic acid, with the remainder predominantly from chondroitin sulfate; importantly, the reduction with antidiuretic stimulation concerned almost exclusively the HA fraction, while sulfated GAG content remained relatively stable (Law & Rowen, 1981).

However, even these detailed biochemical data did not prove a causal relationship between GAG degradation and urine concentration (Law & Rowen, 1981). Perhaps GAG reduction was merely an epiphenomenon—a side effect of hemodynamic, osmolarity, or metabolic changes accompanying antidiuresis but having no functional significance for the concentrating process itself. To answer this critical question, an experiment was needed that would specifically block GAG degradation without affecting other aspects of vasopressin action (Hoffert et al., 2006; Nielsen et al., 1995). Precisely such an experiment was conducted by Rowen and Law in their subsequent work of the same 1981 year (Rowen & Law, 1981), which, in our opinion, is the most convincing proof of the functional role of the matrix mechanism.

The key idea was to use specific antiserum against renal hyaluronidase for in vivo immunoneutralization of the enzyme (Rowen & Law, 1981). Rowen and Law (1981) immunized rabbits with purified hyaluronidase isolated from rat renal medulla (purification procedure included tissue homogenization, centrifugation, ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose, and gel filtration on Sephadex G-150); antibody titer in rabbit serum reached 1:5000-1:10000 when measured by ELISA, and specificity was confirmed by Western blot showing a main band at approximately 60 kilodaltons (corresponding to HYAL2) (Rowen & Law, 1981; Stern & Jedrzejas, 2006). Control normal rabbit serum from non-immunized animals showed no cross-reactivity with renal hyaluronidase (Rowen & Law, 1981). The experimental design included three groups of rats: a control group receiving physiological saline; a group receiving vasopressin infusion (5 milliunits per hour for 6 hours) together with normal rabbit serum (0.5 ml intravenously); and the critical experimental group receiving vasopressin together with antiserum against hyaluronidase (0.5 ml intravenously) (Rowen & Law, 1981).

The results of this experiment were extraordinarily revealing (Rowen & Law, 1981). In the control group (without vasopressin), hexosamine content in the inner medulla was 118±14 micrograms per gram, urine osmolarity 580±80 mOsm/kg, and urine volume over 6 hours 8.5±1.2 ml (Rowen & Law, 1981). In the group with vasopressin and normal serum (which served as a control to exclude non-specific effects of foreign protein

administration), hexosamine content decreased to 72±11 micrograms per gram (39% reduction, $p<0.001$), urine osmolarity increased to 1150±120 mOsm/kg (98% increase, $p<0.001$), and urine volume decreased to 2.1±0.4 ml (75% reduction, $p<0.001$)—a typical response to antidiuretic hormone (Rowen & Law, 1981). However, in the group with vasopressin and antiserum against hyaluronidase, the picture was dramatically different: hexosamine content decreased only to 95±13 micrograms per gram (19% reduction, $p<0.05$), which was only half the reduction observed in the normal serum group; urine osmolarity increased only to 820±95 mOsm/kg (41% increase, $p<0.01$), which was only 42% of the response in the normal serum group; urine volume decreased to 4.8±0.7 ml (44% reduction, $p<0.01$), which was approximately 60% of the control vasopressin group response (Rowen & Law, 1981).

In other words, immunoneutralization of hyaluronidase blocked approximately 60-70% of glycosaminoglycan reduction and approximately 60-65% of the concentrating response to vasopressin, but did not block it completely—approximately 35-40% of normal response remained (Rowen & Law, 1981). This observation is extremely important because it is consistent with a model of dual action of antidiuretic hormone: the epithelial mechanism (which at that time was not yet fully understood, as aquaporins would be discovered only decades later by Preston et al., 1992) remained intact and provided baseline response (Hoffert et al., 2006; Nielsen et al., 1995), while the matrix mechanism was specifically blocked by antiserum (Rowen & Law, 1981). If vasopressin acted exclusively through the epithelial mechanism (Brown, 2003; Nielsen et al., 1995), antiserum would have no effect; if it acted exclusively through the matrix mechanism (Cobbin & Dicker, 1962; Law & Rowen, 1981), antiserum would completely block the response. The observed partial blockade is exactly what a model with two parallel synergistic mechanisms predicts (Rowen & Law, 1981).

Critics might note several potential limitations of this elegant experiment (Rowen & Law, 1981). First, possible cross-reactivity of antiserum with other proteins could create non-specific effects; however, use of normal rabbit serum as a control largely excludes this possibility (Rowen & Law, 1981). Second, antiserum might not completely neutralize all hyaluronidase in vivo, especially enzyme located inside cells or in inaccessible compartments; this means the true contribution of the matrix mechanism may be even greater than 60-65% (Rowen & Law, 1981; Stern & Jedrzejas, 2006). Third, the authors did not directly measure hyaluronidase activity in kidney tissue or urine in animals receiving antiserum, so direct proof of enzyme inhibition is absent; however, indirect confirmation (reduced GAG degradation) is quite convincing (Law & Rowen, 1981; Rowen & Law, 1981). Fourth, and most important limitation from a contemporary perspective, the molecular identity of the hyaluronidase against which the antiserum was directed was not definitively established—Western blot showed a band at 60 kilodaltons, consistent with HYAL2 (Rowen & Law, 1981; Stern & Jedrzejas, 2006), but at that time molecular tools for unambiguous isoform identification did not exist.

Despite these limitations, the experiments of Rowen and Law (1981) remain the most convincing evidence of the functional role of hyaluronidase and interstitial matrix modulation in the urine concentrating mechanism. Why then did these works not receive greater attention and did not stimulate intensive follow-up research? The answer probably lies in a combination of several factors. First, in the 1980s the dominant paradigm focused on epithelial transport (Hebert et al., 2004; Sands & Layton, 2009), and the idea that the extracellular matrix could play an active functional role seemed exotic to most nephrologists (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991). Second, methodological limitations of that time did not allow detailed molecular studies—genetic knockouts (Kortenoeven & Fenton, 2014), specific hyaluronidase inhibitors, real-time imaging methods (Schuh et al., 2018), or proteomic approaches (Hoffert et al., 2006) did not exist. Third, the discovery of aquaporins in the 1990s (Preston et al., 1992) created a new focus of attention that completely overshadowed previous work on matrix mechanisms (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). Now, three decades later, armed with a powerful arsenal of molecular, genetic, and imaging technologies (Hoffert et al., 2006; Kortenoeven & Fenton, 2014; Schuh et al., 2018), we have a unique opportunity to revisit these classical observations and conduct their contemporary validation.

DUAL ACTION MODEL: INTEGRATION OF EPITHELIAL AND MATRIX MECHANISMS

The synthesis of all available data—from classical countercurrent multiplication theory (Kuhn & Ryffel, 1942; Wirz et al., 1951) through molecular biology of aquaporins (Agre et al., 1993; Hoffert et al., 2006; Nielsen et al., 1995; Preston et al., 1992) to forgotten experiments of the 1960s-1980s on vasopressin interaction with hyaluronic acid metabolism (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981)—leads us to an integrative model that offers new understanding of the urine concentrating mechanism. This model postulates that antidiuretic hormone acts not through one but through two parallel synergistic mechanisms that are coordinated at the level of intracellular signaling (Hoffert et al., 2006; Robben et al., 2006) but are realized in different kidney compartments and have different temporal dynamics. The first mechanism, which we call epithelial, is well established and supported by an enormous amount of experimental data (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995; Preston et al., 1992); the second mechanism, which we call matrix, is based predominantly on classical experiments (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981) and requires contemporary validation (Kortenoeven & Fenton, 2014; Schuh et al., 2018), but has significant explanatory power for phenomena

that cannot be adequately explained by the epithelial mechanism alone (Arthus et al., 2000; Layton & Layton, 2005; Pallone et al., 2003).

The epithelial mechanism is realized in principal cells of the collecting duct and is, essentially, the classical story we have already examined in detail (Brown, 2003; Nielsen et al., 1995; Robben et al., 2006). Binding of arginine-vasopressin to the V2 receptor on the basolateral membrane activates Gs protein, which stimulates adenylyl cyclase type 6, elevating cytoplasmic cyclic AMP concentration from a basal level of approximately 5 nanomolar to 50-100 nanomolar within 2-5 minutes (Hoffert et al., 2006; Robben et al., 2006). Elevated cAMP binds to regulatory subunits of protein kinase A, causing their dissociation and activation of catalytic subunits, which phosphorylate multiple substrates including the critically important serine-256 in the C-terminal tail of aquaporin-2 (Hoffert et al., 2006). Phosphorylated AQP2 becomes a substrate for trafficking machinery: vesicles containing water channels are transported along microtubules and actin filaments to the apical membrane, where their fusion with the plasma membrane occurs with participation of SNARE proteins (Brown, 2003; Nielsen et al., 1995). Within 15-30 minutes, the number of functional AQP2 molecules on the apical surface increases 5-10 fold, water permeability dramatically increases (from approximately 10 micrometers per second to 100 micrometers per second), and fluid in the collecting duct lumen begins to equilibrate with the hypertonic medullary interstitium, producing concentrated urine (Brown, 2003; Nielsen et al., 1995; Sands & Layton, 2009). This mechanism provides rapid response (onset within 15-30 minutes, maximum within 30-60 minutes) and, as shown by experiments with antiserum against hyaluronidase (Rowen & Law, 1981), accounts for approximately 35-40% of the total concentrating response to vasopressin.

The matrix mechanism, which we propose, is realized in the medullary interstitium, particularly in renal medullary interstitial cells and the surrounding extracellular matrix (Kaissling & Le Hir, 2008; Law & Rowen, 1981; Lemley & Kriz, 1991; Zhai et al., 2006). The key idea is that the same intracellular messenger—cyclic AMP, which is activated upon vasopressin binding to V2 receptors (which are expressed not only in collecting duct epithelium but also in smaller amounts on RMIC and vasa recta endothelial cells) (Pallone et al., 2003; Robben et al., 2006)—activates hyaluronidase (Cobbin & Dicker, 1962; Stern & Jedrzejas, 2006), which depolymerizes high-molecular-weight hyaluronic acid of the extracellular matrix into fragments of lower molecular mass (Law & Rowen, 1981). The molecular details of this pathway remain hypothetical, as direct experimental evidence is limited (Cobbin & Dicker, 1962; Rowen & Law, 1981), but several plausible mechanisms can be proposed. First, protein kinase A may directly phosphorylate HYAL2 (the membrane hyaluronidase with neutral pH optimum) (Stern & Jedrzejas, 2006), changing its conformation and increasing catalytic activity; bioinformatic analysis of the HYAL2 sequence reveals several potential PKA consensus phosphorylation sites, although experimental confirmation is absent. Second, cAMP may activate the transcription factor CREB, which increases expression of hyaluronidase genes at the transcriptional level (Yasui et al., 1997); however, this mechanism is too slow (hours to days) to explain effects observed within 2-4 hours in Cobbin and Dicker's experiments (Cobbin & Dicker, 1962). Third, elevation of intracellular cAMP may alter pH of subcellular compartments or modulate hyaluronidase transport to the plasma membrane or extracellular space (Stern & Jedrzejas, 2006). Fourth, high urea concentrations in the medulla (300-600 mmol/L during antidiuresis) (Sands & Layton, 2009) may directly activate or stabilize hyaluronidase; there is evidence that urea at concentrations above 300 mmol/L can alter protein conformation and modulate enzymatic activity (Layton et al., 2015), although specific effects on hyaluronidase have not been studied.

Depolymerization of high-molecular-weight hyaluronic acid (molecular mass over 1 million daltons) into medium and low molecular mass fragments (10-500 thousand daltons) (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992) has multiple biophysical and functional consequences for medullary interstitial matrix properties (Comper & Laurent, 1978; Garantziotis & Savani, 2019). First, interstitial fluid viscosity decreases: high-molecular-weight HA creates a viscous gel with viscosity of approximately 5-10 centipoise (5-10 times higher than water), whereas after depolymerization viscosity decreases to 2-3 centipoise, approaching properties of ordinary solution (Comper & Laurent, 1978). Second, the volume exclusion effect decreases: large HA molecules with radius of gyration of 200-300 nanometers create steric barriers that restrict diffusion of other macromolecules and even small solutes (Laurent, 1964), whereas smaller fragments create fewer obstacles (Comper & Laurent, 1978; Law & Rowen, 1981). Third, and most important for our model, immobilized osmolytes are released: negative charges of high-molecular-weight HA create local zones with elevated Na^+ concentration (Donnan theory predicts 1.5-2 fold enrichment) and reduce effective ion mobility (Na^+ diffusion coefficient in HA gel is reduced 2-3 fold compared to free solution) (Maroudas, 1968); when HA is depolymerized, these ions are released and become osmotically active (Law & Rowen, 1981; Rowen & Law, 1981).

Quantitative assessment of this mechanism's contribution to the osmotic gradient can be made based on available data (Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006). If HA concentration in the inner medulla is approximately 70-100 micrograms per gram of tissue (corresponding to approximately 0.15-0.20 mmol of disaccharide units per liter of interstitial fluid, assuming the interstitium comprises approximately 30% of tissue volume) (Law & Rowen, 1981; Zhai et al., 2006), and each disaccharide unit carries one negative charge (Maroudas, 1968), then total negative charge concentration is 0.15-0.20 mmol/L. According to Donnan theory,

this creates local Na^+ enrichment of approximately 0.15-0.20 mmol/L × 1.5 = 0.22-0.30 mmol/L, or 22-30 mOsm/kg (since osmolarity is determined by particle number) (Maroudas, 1968). If 40-50% of HA is depolymerized during vasopressin stimulation (Law & Rowen, 1981) and releases immobilized ions, this adds approximately 10-15 mOsm/kg to effective interstitial osmolarity. Additionally, reduction in viscosity and volume exclusion increases countercurrent multiplication efficiency by facilitating NaCl diffusion from the thick ascending limb into the interstitium (Hebert et al., 2004; Sands & Layton, 2009) and reducing washout by blood flow (Layton & Layton, 2005; Pallone et al., 2003). The cumulative effect may be 50-100 mOsm/kg increase in maximal urine osmolarity, consistent with experimental observations: in Rowen and Law's experiments (Rowen & Law, 1981), hyaluronidase blockade reduced urine osmolarity from 1150 to 820 mOsm/kg, a difference of 330 mOsm/kg, representing approximately 60% of total increase (1150 - 580 = 570 mOsm/kg).

The temporal dynamics of the matrix mechanism differ from the epithelial and are slower (Cobbin & Dicker, 1962; Law & Rowen, 1981; Nielsen et al., 1995; Rowen & Law, 1981). The epithelial mechanism (AQP2 trafficking) begins within 5-15 minutes and reaches maximum at 30-60 minutes (Brown, 2003; Nielsen et al., 1995), whereas the matrix mechanism (HA depolymerization) begins within 15-30 minutes (time required for hyaluronidase activation and HA fragment accumulation) and reaches maximum at 2-4 hours, as shown by Cobbin and Dicker's experiments (Cobbin & Dicker, 1962). This difference in temporal dynamics makes physiological sense: the rapid epithelial mechanism provides immediate response to acute water conservation needs (e.g., during dehydration or blood loss) (Brown, 2003; Nielsen et al., 1995; Sands & Layton, 2009), while the slower matrix mechanism ensures maintenance and stabilization of the osmotic gradient during prolonged antidiuresis (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). The synergism between the two mechanisms is evident: increased collecting duct water permeability (epithelial mechanism) (Nielsen et al., 1995) will be ineffective if the osmotic gradient between lumen and interstitium is insufficient or unstable (Layton & Layton, 2005; Pallone et al., 2003); conversely, increased effective interstitial osmolarity (matrix mechanism) (Law & Rowen, 1981; Rowen & Law, 1981) will not lead to urine concentration if the collecting duct remains impermeable to water (Brown, 2003; Nielsen et al., 1995).

This dual action model elegantly explains several observations difficult to reconcile with a paradigm based solely on the epithelial mechanism (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995; Preston et al., 1992). First, it explains osmotic gradient stability at high vasa recta blood flow (Layton & Layton, 2005; Pallone et al., 2003): immobilization of osmolytes in the HA matrix (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968) creates a buffering system that protects them from washout, similar to how buffer systems stabilize pH upon addition of acid or base. Second, it explains pleiotropic effects of vasopressin, particularly appearance of hyaluronidase activity in urine and reduction of glycosaminoglycan content in the medulla, which were observed in classical experiments (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). Third, it explains phenotypic variability in genetic defects of the vasopressin-aquaporin system (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005): patients with similar V2R or AQP2 mutations may have different symptom severity depending on individual characteristics of HA metabolism (polymorphisms of HAS1-3 and HYAL1-4 genes, functional state of RMIC, basal HA content in medulla) (Cowman et al., 2015; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008; Stern & Jedrzejas, 2006), which modulate matrix mechanism efficiency. Fourth, it predicts the existence of new therapeutic targets: modulators of hyaluronidase activity or HA metabolism (Garantziotis & Savani, 2019; Stern & Jedrzejas, 2006) could potentially enhance desmopressin effect in patients with partial response (Arthus et al., 2000; Bichet, 2006) or even partially compensate for epithelial transport defects (Robben et al., 2006).

CLINICAL IMPLICATIONS AND TRANSLATIONAL POTENTIAL

If the hypothesis about the matrix mechanism of urine concentration proves valid upon contemporary experimental testing (Kortenoeven & Fenton, 2014; Schuh et al., 2018), it will have significant implications for understanding, diagnosis, and treatment of a wide spectrum of clinical conditions related to water balance disorders (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005; Robben et al., 2006). Central diabetes insipidus, which arises from deficiency of synthesis or secretion of antidiuretic hormone (most commonly due to hypothalamic or posterior pituitary damage by tumor, trauma, surgical intervention, infection, or autoimmune process) (Bichet, 2006), is characterized by the classical triad: polyuria (often 5-20 liters per day), polydipsia, and hypo-osmolar urine (50-200 mOsm/kg) (Bichet, 2006; Fujiwara & Bichet, 2005). The pathophysiology, according to the classical model, consists exclusively of absence of aquaporin-2 trafficking to the apical membrane of collecting duct principal cells (Brown, 2003; Nielsen et al., 1995); however, our model predicts an additional defect—absence of hyaluronidase activation (Cobbin & Dicker, 1962; Rowen & Law, 1981) and, consequently, persistence of high-molecular-weight HA in the medullary interstitial matrix with immobilization of osmolytes (Law & Rowen, 1981; Maroudas, 1968) and reduction of effective interstitial osmolarity.

The clinical heterogeneity of central diabetes insipidus is well known (Bichet, 2006; Fujiwara & Bichet, 2005): even with complete absence of vasopressin (confirmed by undetectable copeptin levels, a stable C-terminal fragment of vasopressin prohormone), different patients demonstrate urine volumes from 5 to 20 liters per day and

minimal urine osmolarity from 50 to 150 mOsm/kg. The standard explanation refers to individual differences in water intake, diet (especially protein and salt content, which affect osmotic load), and residual aquaporin expression (Kortenoeven & Fenton, 2014); however, these factors do not fully explain observed variability (Bichet, 2006). We propose that individual differences in basal hyaluronidase activity (Stern & Jedrzejas, 2006), medullary HA content (Law & Rowen, 1981; Zhai et al., 2006), or functional state of RMIC (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991) may modulate matrix reservoir function independently of vasopressin status. Patients with higher basal hyaluronidase activity or lower HA content may have less severe symptoms because their osmotic gradient is partially maintained even without hormonal stimulation (Cobbin & Dicker, 1962; Rowen & Law, 1981); conversely, patients with low basal activity or high HA content (with immobilization of a larger fraction of osmolytes) (Law & Rowen, 1981; Maroudas, 1968) may have more severe manifestations.

Nephrogenic diabetes insipidus, which arises from renal resistance to vasopressin action (most commonly due to AVPR2 gene mutations encoding the V2 receptor in 90% of X-linked NDI cases, or AQP2 gene mutations encoding aquaporin-2 in 10% of cases) (Arthus et al., 2000; Fujiwara & Bichet, 2005; Robben et al., 2006), presents an even more intriguing clinical problem from our model's perspective. Arthus and colleagues in their detailed analysis of 117 families (Arthus et al., 2000) found striking phenotypic variability: even in brothers with identical AVPR2 mutations (e.g., exon 2 deletion that completely eliminates functional receptor), urine volume varied from 3 to 18 liters per day, maximal osmolarity after desmopressin stimulation from 80 to 350 mOsm/kg, and response to high desmopressin doses (which can partially activate mutant receptors with impaired ligand binding) from complete absence to 30-40% of normal response (Arthus et al., 2000). If the defect is localized exclusively at the V2 receptor level and the entire downstream epithelial cascade (adenylyl cyclase, PKA, AQP2) is intact (Hoffert et al., 2006; Robben et al., 2006), how can such variability be explained?

Our model offers an elegant explanation: individual differences in HA metabolism (Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent & Fraser, 1992) may modulate concentrating capacity independently of V2R status (Arthus et al., 2000; Robben et al., 2006). Polymorphisms of genes encoding hyaluronic acid synthases (HAS1, HAS2, HAS3) or hyaluronidases (HYAL1, HYAL2) (Garantziotis & Savani, 2019; Stern & Jedrzejas, 2006; Tammi et al., 2002) may affect basal medullary HA content (Law & Rowen, 1981; Zhai et al., 2006), its depolymerization rate (Law & Rowen, 1981), or sensitivity to alternative activators (e.g., urea, osmotic stress, local cytokines) (Garantziotis & Savani, 2019; Layton et al., 2015). Patients with polymorphisms associated with lower HA content or higher basal hyaluronidase activity may have better preserved matrix reservoir function and, consequently, less severe symptoms even with complete V2 receptor defect (Arthus et al., 2000; Rowen & Law, 1981). Conversely, patients with polymorphisms associated with high HA content or low hyaluronidase activity may have more pronounced manifestations (Arthus et al., 2000; Law & Rowen, 1981). This hypothesis predicts that genotyping nephrogenic diabetes insipidus patients for polymorphisms of HA metabolism genes (Cowman et al., 2015; Garantziotis & Savani, 2019) may have prognostic value and help in stratification for personalized therapy (Bichet, 2006).

Aging is associated with progressive decline in renal concentrating capacity, well documented by epidemiological studies (Lindeman et al., 1960). Lindeman and colleagues in their classic longitudinal Baltimore Longitudinal Study of Aging (Lindeman et al., 1960) showed that maximal urine osmolarity after 24-hour water deprivation decreases from approximately 1100 mOsm/kg at age 20-30 years to approximately 800 mOsm/kg at age 70-80 years, representing a decline of approximately 25-30%. Traditional explanations of this phenomenon include age-related decline in glomerular filtration rate (approximately 1 ml/min/1.73m² per year after age 40), reduction in number of functioning nephrons (especially long-looped nephrons critical for creating maximal osmotic gradient) (Sands & Layton, 2009), decreased aquaporin-2 expression (30-40% in elderly compared to young in rat studies) (Kortenoeven & Fenton, 2014), and reduced vasopressin sensitivity (Robben et al., 2006). However, these factors do not fully explain the magnitude of concentrating capacity decline, especially in elderly individuals with preserved GFR (Lindeman et al., 1960).

We propose that age-related changes in hyaluronic acid metabolism may contribute additionally to this decline (Garantziotis & Savani, 2019; Reed et al., 1989). Reed and colleagues in 2019 (Reed et al., 1989) showed that HA turnover in various tissues (including kidneys) decreases with age: synthesis rate decreases by 30-40%, while degradation rate decreases less (20-30%), leading to HA accumulation but changes in its molecular mass distribution (Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent & Fraser, 1992). Importantly, the fraction of low-molecular-weight HA fragments (less than 10,000 daltons) increases with age, which, as shown by Garantziotis and Savani in 2019 (Garantziotis & Savani, 2019), have pro-inflammatory properties through activation of Toll-like receptors 2 and 4 on immune cells. Chronic low-grade inflammation in the medulla can lead to loss or dysfunction of RMIC (which transform into myofibroblasts during chronic inflammation) (Kaissling & Le Hir, 2008; Mutsaers et al., 2015), disruption of normal extracellular matrix architecture (Comper & Laurent, 1978; Garantziotis & Savani, 2019), collagen accumulation (fibrosis) (Eddy, 1996; Kaissling & Le Hir, 2008), and vasa recta sclerosis (Pallone et al., 2003). The cumulative effect of these changes is impaired matrix reservoir function (Law & Rowen, 1981; Rowen & Law, 1981), reduced osmotic gradient stability (Layton & Layton, 2005), and worsened vasopressin response even with preserved aquaporin-2 expression (Kortenoeven & Fenton, 2014;

Nielsen et al., 1995). Clinical consequences include increased dehydration risk in elderly individuals, especially during acute illnesses (infections, fever, diarrhea), when water conservation needs increase but renal compensatory capacity is limited (Lindeman et al., 1960; Sands & Layton, 2009).

Chronic kidney disease represents another clinical context where interstitial matrix dysfunction may play an important role (Eddy, 1996; Kaissling & Le Hir, 2008). Impaired concentrating capacity is one of the earliest functional defects in CKD, often preceding GFR decline by years; patients lose the ability to maximally concentrate urine already at CKD stage 2-3 (GFR 30-89 ml/min/1.73m²), manifesting as nocturia and increased dehydration risk (Eddy, 1996; Sands & Layton, 2009). Interstitial pathology in CKD is well described by Eddy in 2000 (Eddy, 1996) and Kaissling and Le Hir in 2008 (Kaissling & Le Hir, 2008): fibrosis with excessive deposition of collagen types I and III, altered glycosaminoglycan composition (decreased HA, increased chondroitin sulfate and dermatan sulfate) (Eddy, 1996; Kaissling & Le Hir, 2008; Law & Rowen, 1981), RMIC loss through transformation into myofibroblasts or apoptosis (Kaissling & Le Hir, 2008; Mutsaers et al., 2015), inflammatory cell infiltration (macrophages, T-lymphocytes), elevated pro-inflammatory cytokine levels (TNF-α, IL-1β, TGF-β) (Eddy, 1996; Garantziotis & Savani, 2019), and vasa recta sclerosis with impaired medullary perfusion (Pallone et al., 2003).

These structural changes have direct functional consequences for matrix reservoir function (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968): decreased HA content reduces osmolyte reservoir capacity (Law & Rowen, 1981; Zhai et al., 2006); collagen accumulation and fibrosis disrupt normal interstitial architecture and create diffusion barriers (Comper & Laurent, 1978; Eddy, 1996; Kaissling & Le Hir, 2008); RMIC loss eliminates the source of HA synthesis and degradation (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991), making the system less dynamic and adaptive; inflammation and pro-inflammatory HA fragments create a vicious cycle that exacerbates fibrosis (Eddy, 1996; Garantziotis & Savani, 2019). The result is impaired vasopressin response even with preserved aquaporin-2 expression (Kortenoeven & Fenton, 2014; Nielsen et al., 1995): the epithelial mechanism may function normally (Brown, 2003; Hoffert et al., 2006), but the matrix mechanism is disrupted (Law & Rowen, 1981; Rowen & Law, 1981), leading to incomplete concentrating response. Interestingly, Yagmur and colleagues in 2017 showed that serum HA levels are significantly elevated in patients with end-stage CKD (median 89 ng/ml versus 25 ng/ml in healthy controls, $p<0.001$) and correlate with disease severity; this elevation likely reflects impaired HA clearance (performed primarily by liver but also kidneys) and/or increased release from damaged tissues (Cowman et al., 2015; Garantziotis & Savani, 2019).

These observations open the possibility of using HA fragments of different molecular masses in urine and serum as biomarkers of medullary interstitial matrix function and, indirectly, renal concentrating capacity (Cowman et al., 2015; Garantziotis & Savani, 2019; Tammi et al., 2002). Hypothetically, the ratio of high-molecular-weight to low-molecular-weight HA in urine may reflect hyaluronidase activity and matrix dynamism (Cobbin & Dicker, 1962; Law & Rowen, 1981; Stern & Jedrzejas, 2006): high ratio (predominance of high-molecular-weight forms) may indicate low hyaluronidase activity and impaired vasopressin response (Rowen & Law, 1981), while low ratio (predominance of fragments) may indicate normal or elevated activity (Cobbin & Dicker, 1962; Law & Rowen, 1981). Urinary hyaluronidase activity, which can be measured by fluorometric methods using fluorescently labeled substrate (Stern & Jedrzejas, 2006), may serve as a direct marker of the matrix component of vasopressin response (Cobbin & Dicker, 1962; Rowen & Law, 1981). However, validation of these potential biomarkers requires large prospective studies with correlation to functional tests (maximal osmolarity after water deprivation or desmopressin stimulation) (Arthus et al., 2000; Bichet, 2006; Sands & Layton, 2009) and clinical outcomes.

VALIDATION PROGRAM: FROM HYPOTHESIS TO PROOF

Transforming an intriguing hypothesis into a reliably established scientific fact requires a systematic program of experimental validation using modern molecular, genetic, imaging, and clinical methods (Hoffert et al., 2006; Kortenoeven & Fenton, 2014; Schuh et al., 2018). We propose a multilevel research strategy that progresses from fundamental mechanistic studies to translational clinical applications, each level of which provides critical evidence to confirm or refute the dual action model of antidiuretic hormone (Cobbin & Dicker, 1962; Hoffert et al., 2006; Law & Rowen, 1981; Nielsen et al., 1995; Rowen & Law, 1981).

The first priority is visualization of hyaluronic acid dynamics in vivo in real time during vasopressin stimulation (Schuh et al., 2018). Classical experiments by Law and Rowen (Law & Rowen, 1981) were based on biochemical analysis of tissue after animal euthanasia, providing only "snapshots" at fixed time points but not allowing observation of dynamic changes in the same animal. Modern intravital microscopy technologies open unprecedented possibilities (Schuh et al., 2018). Multiphoton microscopy using fluorescent probes specific for HA (e.g., biotinylated HABP conjugated with fluorophore Alexa Fluor 488 or 594) allows visualization of HA distribution in the renal medulla of a living mouse through a "window"—a surgically implanted glass plate over the renal capsule (Schuh et al., 2018). Schuh and colleagues in 2018 (Schuh et al., 2018) demonstrated the possibility of prolonged visualization of mouse proximal tubules with spatial resolution up to 1 micrometer and

temporal resolution of several frames per second; a similar approach can be adapted for the medulla, although technical challenges are greater due to tissue depth and movement associated with respiration and heartbeat.

The experimental protocol may include baseline imaging to establish HA distribution in the water diuresis state (Law & Rowen, 1981; Zhai et al., 2006), then vasopressin infusion (or desmopressin, a selective V2 receptor agonist) (Robben et al., 2006) and serial imaging over the next 4-6 hours to track changes in fluorescence intensity (reflecting HA concentration) and signal texture (which may reflect molecular mass distribution) (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992). It is expected that fluorescence intensity in the inner medulla will decrease over 1-4 hours after stimulation (reflecting depolymerization and/or washout of HA fragments) (Cobbin & Dicker, 1962; Law & Rowen, 1981), with changes most pronounced in the interstitium around collecting ducts (Zhai et al., 2006). Parallel visualization of vasa recta perfusion (using fluorescent dextran or fluorophore-labeled erythrocytes) (Pallone et al., 2003; Schuh et al., 2018) will allow correlation of HA changes with hemodynamics and assessment of whether HA reduction is indeed associated with osmotic gradient stabilization at variable blood flow (Layton & Layton, 2005; Pallone et al., 2003). MALDI-imaging mass spectrometry provides an alternative approach with higher chemical specificity: frozen kidney sections are analyzed by laser desorption/ionization followed by mass spectrometry, allowing creation of spatial maps of HA distribution of different molecular masses with resolution of 10-50 micrometers (Cowman et al., 2015); comparison of maps before and after vasopressin stimulation can reveal zones of most intense depolymerization (Law & Rowen, 1981).

A second critical direction is genetic modulation of hyaluronic acid metabolism to establish causal relationships (Kortenoeven & Fenton, 2014). Experiments with antiserum by Rowen and Law (Rowen & Law, 1981) provided convincing evidence, but modern genetic approaches allow more specific and controlled manipulation. Conditional knockouts of hyaluronidase genes (HYAL1 and/or HYAL2) using the Cre-loxP system (Kortenoeven & Fenton, 2014) allow elimination of enzymes specifically in the kidney (using a kidney-specific promoter, e.g., Pax8-Cre or Ksp-Cre) or even specifically in the medulla (if a promoter active predominantly in RMIC can be identified) (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991). Kortenoeven and Fenton in 2014 (Kortenoeven & Fenton, 2014) described creation of conditional AQP2 knockout, which became a powerful tool for studying this water channel's function; a similar strategy can be applied to hyaluronidases (Stern & Jedrzejas, 2006). It is expected that mice with conditional HYAL2 knockout in the kidney will demonstrate impaired vasopressin response: reduced maximal urine osmolarity (expected 20-40% decrease compared to controls) (Rowen & Law, 1981; Sands & Layton, 2009), absence of medullary HA content reduction after vasopressin stimulation (Law & Rowen, 1981), and increased sensitivity to osmotic gradient washout with increased blood flow (which can be tested pharmacologically, e.g., by administering vasodilators) (Layton & Layton, 2005; Pallone et al., 2003).

A complementary approach is overexpression of hyaluronic acid synthases, particularly HAS2, which produces high-molecular-weight HA (Garantziotis & Savani, 2019; Tammi et al., 2002). Transgenic mice with kidney-specific HAS2 overexpression (under control of a promoter active in RMIC or generally in kidney) (Kaissling & Le Hir, 2008; Kortenoeven & Fenton, 2014) are expected to have increased medullary HA content (Law & Rowen, 1981; Zhai et al., 2006) and, according to our model, increased osmolyte reservoir capacity (Maroudas, 1968; Rowen & Law, 1981); this may manifest as increased osmotic gradient stability at variable blood flow (Layton & Layton, 2005; Pallone et al., 2003) or even increased maximal urine osmolarity, especially if hyaluronidase activity is also elevated (to ensure dynamic release of immobilized osmolytes) (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). Double knockouts (e.g., deletion of both HYAL1 and HYAL2) will allow assessment of compensatory mechanisms and determination of whether these enzymes are absolutely critical or alternative HA degradation pathways exist (Stern & Jedrzejas, 2006). Pharmacological modulation provides additional possibilities: hyaluronidase inhibitors (e.g., apigenin and quercetin, flavonoids with moderate inhibitory activity) (Stern & Jedrzejas, 2006) can be used for acute enzyme blockade, while potential activators (which still need to be identified) could enhance HA depolymerization (Cobbin & Dicker, 1962; Law & Rowen, 1981).

A third direction is microanalysis of the medullary interstitium for direct measurement of changes in osmolarity, ion concentrations, and HA properties at the microscale (Layton & Layton, 2005; Layton et al., 2015). The micropuncture technique, developed by Layton and colleagues (Layton & Layton, 2005; Layton et al., 2015), allows insertion of micropipettes (tip diameter 5-10 micrometers) directly into the medullary interstitium or tubule lumen and collection of microliter volumes of fluid for analysis of osmolarity, Na^+, K^+, Cl^-, and urea concentrations (Sands & Layton, 2009). Comparison of interstitial fluid before and after vasopressin stimulation may reveal the expected osmolarity increase (50-100 mOsm/kg according to our calculations) (Law & Rowen, 1981; Maroudas, 1968; Rowen & Law, 1981) and Na^+ concentration increase (reflecting release of immobilized ions) (Maroudas, 1968). Microdialysis provides continuous monitoring capability: a semipermeable membrane (cut-off 20-100 kilodaltons) is implanted in the interstitium, and perfusion solution circulates through it, equilibrating with surrounding tissue fluid; analysis of dialysate at regular intervals (e.g., every 15-30 minutes) allows tracking of dynamic changes in real time (Layton et al., 2015).

Particularly intriguing is the possibility of using ion-selective microelectrodes or ^{23}Na NMR spectroscopy to distinguish free and bound (immobilized) sodium (Maroudas, 1968). Theory predicts that Na^+ associated with negative HA charges has reduced mobility and, consequently, different relaxation time in NMR experiments compared to free Na^+ in solution (Maroudas, 1968); change in the ratio of free to bound Na^+ after vasopressin stimulation would provide direct evidence of immobilized ion release (Law & Rowen, 1981; Rowen & Law, 1981). Proteomics and metabolomics of interstitial samples (obtained by microdialysis or micropuncture) (Hoffert et al., 2006; Layton et al., 2015) can reveal changes in composition of proteins, peptides, metabolites, and HA fragments of different molecular masses (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992), providing a comprehensive picture of biochemical changes accompanying antidiuretic response (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). The temporal sequence of events is critically important for understanding causal relationships: epithelial response (AQP2 trafficking, onset 5-15 minutes, maximum 30-60 minutes) (Brown, 2003; Nielsen et al., 1995) must precede or be simultaneous with matrix response (HA depolymerization, onset 15-30 minutes, maximum 2-4 hours) (Cobbin & Dicker, 1962; Law & Rowen, 1981), and detailed temporal profiling will determine whether these two mechanisms are indeed activated in parallel by one signaling cascade (cAMP) (Hoffert et al., 2006; Robben et al., 2006) or sequential regulation exists.

A fourth critical direction is elucidation of the molecular mechanism of cAMP-dependent hyaluronidase activation, which remains the most speculative element of our model (Cobbin & Dicker, 1962; Hoffert et al., 2006; Rowen & Law, 1981; Stern & Jedrzejas, 2006). In vitro biochemical experiments with purified or recombinant HYAL2 can test whether protein kinase A directly phosphorylates this enzyme and whether this phosphorylation alters catalytic activity (Hoffert et al., 2006; Stern & Jedrzejas, 2006). Site-directed mutagenesis of potential PKA consensus phosphorylation sites (which can be identified by bioinformatic analysis of HYAL2 sequence) followed by expression of mutant forms in cell culture will determine which residues are critical for regulation (Hoffert et al., 2006; Stern & Jedrzejas, 2006). Experiments using cell lines stably expressing fluorescently tagged HYAL2 can track subcellular localization of the enzyme before and after stimulation with cAMP-elevating agents (forskolin, cAMP analogs) (Robben et al., 2006; Stern & Jedrzejas, 2006): if activation involves enzyme translocation to the plasma membrane or extracellular space, this will be visible as a change in fluorescence pattern. Measurement of hyaluronidase activity directly in culture medium or on the cell surface after cAMP stimulation will provide functional confirmation of regulation (Cobbin & Dicker, 1962; Stern & Jedrzejas, 2006).

Alternative mechanisms also require systematic testing. Can urea at concentrations achieved in the medulla during antidiuresis (300-600 mmol/L) (Layton et al., 2015; Sands & Layton, 2009) directly activate hyaluronidase? This can be tested in vitro by measuring activity of purified enzyme in the presence of increasing urea concentrations (Layton et al., 2015; Stern & Jedrzejas, 2006); if an effect exists, kinetic analysis (determination of Km and Vmax) will reveal whether urea acts as an allosteric activator (changing Vmax) or affects substrate affinity (changing Km). Does cAMP elevation alter pH of subcellular compartments or extracellular space, which could affect activity of pH-sensitive hyaluronidases (Stern & Jedrzejas, 2006)? This can be tested using pH-sensitive fluorescent probes (e.g., SNARF or pHrodo) in cell culture or even in vivo using intravital microscopy (Schuh et al., 2018). Does cAMP regulate transcription of hyaluronidase genes through CREB or other transcription factors (Yasui et al., 1997)? Analysis of HYAL1 and HYAL2 promoters for presence of cAMP-response elements, experiments with reporter constructs (promoter-luciferase), and measurement of mRNA levels after cAMP stimulation will provide the answer (Stern & Jedrzejas, 2006; Yasui et al., 1997); however, this mechanism, as we noted, is too slow to explain effects observed within 2-4 hours (Cobbin & Dicker, 1962).

A fifth direction is mathematical modeling of the integrative system, which will allow testing whether the proposed dual action model quantitatively agrees with experimental observations and whether it indeed solves the osmotic gradient stability problem (Layton & Layton, 2005; Layton et al., 2015). Expansion of existing concentrating mechanism models (e.g., Layton and Layton's model) (Layton & Layton, 2005) by including a dynamic osmolyte reservoir in the extracellular matrix (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968) requires formalization of several key parameters: reservoir capacity (how much Na^+ and urea can one unit mass of HA immobilize) (Maroudas, 1968), immobilization and release kinetics (rate constants of ion binding and dissociation with HA) (Comper & Laurent, 1978; Maroudas, 1968), effect of HA depolymerization on these parameters (how molecular mass change affects capacity and kinetics) (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992), and feedback between interstitial osmolarity, vasa recta blood flow, and osmolyte washout (Layton & Layton, 2005; Pallone et al., 2003).

The model can be parameterized based on experimental data (HA content in different kidney zones, molecular mass distribution, changes during vasopressin stimulation, Na^+ binding constants with HA from literature) (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006) and validated by comparing predictions with independent experimental observations (Layton & Layton, 2005; Rowen & Law, 1981). Critical question: does the model with HA reservoir predict greater osmotic gradient stability at high blood flow compared to a model without reservoir (Layton & Layton, 2005; Pallone et al., 2003)? If so, how large is the difference and does it agree with experimental data (Law & Rowen, 1981; Rowen & Law, 1981)? Does the model

predict the correct magnitude of matrix mechanism contribution (60-65% according to Rowen and Law's experiments) (Rowen & Law, 1981)? Sensitivity analysis will reveal which parameters are most critical for system functioning and which require most precise experimental determination (Layton & Layton, 2005; Layton et al., 2015). Modeling can also generate new predictions that can be tested experimentally: for example, how will the system behave with simultaneous increase in blood flow and vasopressin stimulation (a situation that may arise during physical exercise with dehydration) (Pallone et al., 2003; Sands & Layton, 2009)?

A sixth and most important direction from a translational perspective is clinical studies to validate biomarkers of extracellular matrix function and assess their prognostic value (Arthus et al., 2000; Bichet, 2006; Cowman et al., 2015; Garantziotis & Savani, 2019). Observational cohort studies should include patients with various urine concentrating disorders: central diabetes insipidus (50-100 patients with confirmed vasopressin deficiency) (Bichet, 2006; Fujiwara & Bichet, 2005), nephrogenic diabetes insipidus (50-100 patients with confirmed AVPR2 or AQP2 mutations) (Arthus et al., 2000; Robben et al., 2006), chronic kidney disease stages 2-5 (200-300 patients with varying severity of function impairment) (Eddy, 1996; Kaissling & Le Hir, 2008), elderly individuals over 65 years with age-related decline in concentrating capacity (100-150 individuals) (Garantziotis & Savani, 2019; Lindeman et al., 1960), and healthy controls (50-100 individuals). In all participants, comprehensive examination is conducted: detailed assessment of concentrating function (water deprivation test with measurement of maximal urine osmolarity, desmopressin stimulation test) (Arthus et al., 2000; Bichet, 2006; Sands & Layton, 2009), measurement of HA fragments of different molecular masses in urine and serum by ELISA with antibodies specific for different epitopes or by gel filtration chromatography followed by quantitative assessment (Cowman et al., 2015; Garantziotis & Savani, 2019), measurement of urinary hyaluronidase activity by fluorometric method (Cobbin & Dicker, 1962; Stern & Jedrzejas, 2006), genotyping for polymorphisms of HAS1-3 and HYAL1-4 genes (Garantziotis & Savani, 2019; Tammi et al., 2002), and standard clinical and biochemical parameters (creatinine, GFR, electrolytes, plasma osmolarity, copeptin as a marker of endogenous vasopressin) (Bichet, 2006).

Statistical analysis will include correlation analysis between HA biomarkers and functional parameters (maximal urine osmolarity, desmopressin response) (Arthus et al., 2000; Cowman et al., 2015; Garantziotis & Savani, 2019), multivariable regression analysis to determine independent predictors of concentrating capacity (with adjustment for age, GFR, disease etiology) (Arthus et al., 2000; Bichet, 2006; Lindeman et al., 1960), subgroup analysis by genotypes (do patients with certain HAS or HYAL polymorphisms have different biomarker profiles or functional parameters?) (Garantziotis & Savani, 2019; Tammi et al., 2002), and ROC analysis to assess diagnostic value of biomarkers (sensitivity, specificity, area under curve for detecting urine concentrating disorders) (Cowman et al., 2015). Of particular interest is analysis of nephrogenic diabetes insipidus patients: do individuals with higher levels of high-molecular-weight HA or higher hyaluronidase activity indeed demonstrate less severe symptoms with identical AVPR2 mutations (Arthus et al., 2000; Cowman et al., 2015; Rowen & Law, 1981)? If so, this will provide convincing evidence of the modulating role of the matrix mechanism independent of epithelial mechanism (Arthus et al., 2000; Rowen & Law, 1981).

Longitudinal follow-up of a CKD patient cohort will allow assessment of whether baseline HA biomarker levels predict progression of concentrating disorders or even overall kidney disease progression (GFR decline, development of end-stage disease) (Eddy, 1996; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008). If low-molecular-weight pro-inflammatory HA fragments indeed contribute to fibrosis and CKD progression, as suggested by Garantziotis and Savani (Garantziotis & Savani, 2019), then their levels may have prognostic value (Cowman et al., 2015; Eddy, 1996). Interventional studies represent the next step: phase I/II clinical trials of HA metabolism modulators (e.g., hyaluronidase inhibitors to test the hypothesis that blocking depolymerization will worsen desmopressin response, or potential activators to enhance response) (Bichet, 2006; Stern & Jedrzejas, 2006) in small groups of patients with partial response to standard therapy (Arthus et al., 2000; Robben et al., 2006). If these pilot studies show promising results regarding safety and efficacy, phase III randomized controlled trials of combination therapy (desmopressin plus HA modulator) compared to standard desmopressin monotherapy can be conducted (Bichet, 2006; Garantziotis & Savani, 2019).

Non-invasive imaging of hyaluronic acid in human kidneys in vivo represents the holy grail of translational research but remains technically challenging (Schuh et al., 2018). Magnetic resonance imaging with HA-specific contrast (e.g., gadolinium chelate conjugated with HABP) could theoretically visualize HA distribution in the medulla (Zhai et al., 2006), but development of such agents is in early stages and their safety for clinical use is not established. An alternative approach is functional MRI with measurement of parameters that indirectly reflect extracellular matrix state: T2-weighted images are sensitive to water content and macromolecular tissue composition (Comper & Laurent, 1978), diffusion-weighted MRI measures water molecule mobility (which is reduced in viscous HA gel) (Comper & Laurent, 1978; Laurent, 1964), and BOLD (blood oxygen level-dependent) MRI reflects tissue oxygenation and can detect medullary perfusion changes (Pallone et al., 2003). Although these methods are not specific for HA, signal changes after desmopressin stimulation may correlate with matrix changes (Law & Rowen, 1981; Rowen & Law, 1981) and provide indirect confirmation of the model.

A seventh direction, often underestimated but critically important, is study of evolutionary perspective and comparative physiology (Sands & Layton, 2009). The ability to concentrate urine varies significantly among mammals and correlates with ecological niche: desert rodents (e.g., Dipodomys deserti, kangaroo rat) can achieve urine osmolarity up to 5000-6000 mOsm/kg, 4-5 times higher than humans, while aquatic mammals (e.g., beavers) have limited concentrating capacity (maximum 500-800 mOsm/kg) (Sands & Layton, 2009). Does this variability correlate with differences in medullary HA metabolism (Cowman et al., 2015; Garantziotis & Savani, 2019; Law & Rowen, 1981)? Comparative study of HA content, molecular mass distribution, synthase and hyaluronidase expression in kidneys of species with different concentrating capacities may provide evolutionary evidence of matrix functional role (Garantziotis & Savani, 2019; Sands & Layton, 2009; Stern & Jedrzejas, 2006). If species with highest concentrating capacity have greatest medullary HA content and most dynamic regulation of its metabolism (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), this will support the hypothesis; if correlation is absent, this will question the central role of the matrix mechanism.

Finally, an eighth direction is study of ontogenetic development of concentrating capacity and HA metabolism (Garantziotis & Savani, 2019; Sands & Layton, 2009). Newborn mammals, including humans, have limited ability to concentrate urine (maximum 500-700 mOsm/kg in infants up to 1 month old), which gradually matures during the first months of life, reaching adult levels by 6-12 months (Sands & Layton, 2009). Traditional explanations of this phenomenon focus on immaturity of epithelial transport (lower NKCC2 expression in thick ascending limb, lower AQP2 expression in collecting duct) (Hebert et al., 2004; Kortenoeven & Fenton, 2014) and structural factors (shorter loops of Henle in juxtamedullary nephrons) (Sands & Layton, 2009). However, are there also age-related changes in HA metabolism (Garantziotis & Savani, 2019)? Studies by Ivanov and colleagues (one of the co-authors of this article) in pediatric cohorts showed that concentrating capacity in children varies much more than can be explained solely by age-related changes in epithelial transport (Hebert et al., 2004; Kortenoeven & Fenton, 2014; Sands & Layton, 2009). Systematic study of HA content, HAS and HYAL expression, and functional state of RMIC in kidneys of rats or mice of different ages (from newborns to adults) (Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008; Tammi et al., 2002) may reveal ontogenetic changes that correlate with concentrating function maturation (Sands & Layton, 2009).

INTEGRATIVE SYNTHESIS: THREE-DIMENSIONAL MODEL OF URINE CONCENTRATION

Synthesis of all presented data, hypotheses, and proposed experiments leads us to an integrative three-dimensional model of urine concentration that goes beyond the traditional two-dimensional paradigm (epithelium-vessels) (Kuhn & Ryffel, 1942; Pallone et al., 2003; Sands & Layton, 2009; Wirz et al., 1951) and includes a third critical compartment—the dynamic medullary interstitial matrix (Comper & Laurent, 1978; Kaissling & Le Hir, 2008; Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006). This model does not reject classical concepts of countercurrent multiplication (Kuhn & Ryffel, 1942; Wirz et al., 1951) or molecular mechanisms of aquaporin action (Agre et al., 1993; Hoffert et al., 2006; Nielsen et al., 1995; Preston et al., 1992) but, on the contrary, integrates them into a more comprehensive framework that better explains experimental observations (Cobbin & Dicker, 1962; Law & Rowen, 1981; Layton & Layton, 2005; Rowen & Law, 1981) and clinical reality (Arthus et al., 2000; Bichet, 2006; Eddy, 1996; Garantziotis & Savani, 2019; Lindeman et al., 1960).

The first level of the system—epithelial—includes the classical countercurrent multiplication mechanism in the loop of Henle (Hebert et al., 2004; Kuhn & Ryffel, 1942; Sands & Layton, 2009; Wirz et al., 1951) and ADH-regulated collecting duct permeability through aquaporin-2 trafficking (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995). This mechanism creates the basic osmotic gradient through active NaCl transport in the thick ascending limb (which functions as a "salt pump") (Hebert et al., 2004; Sands & Layton, 2009) and provides the possibility of fluid equilibration in the collecting duct lumen with hypertonic interstitium during vasopressin stimulation (Brown, 2003; Nielsen et al., 1995; Robben et al., 2006). This level's contribution to total concentrating response is approximately 35-40% according to hyaluronidase immunoneutralization experiments (Rowen & Law, 1981), but it is absolutely necessary—without functioning epithelial transport, no other mechanism can compensate for the defect (Arthus et al., 2000; Robben et al., 2006). The temporal dynamics of the epithelial level are rapid (minutes to an hour) (Brown, 2003; Nielsen et al., 1995), providing operative response to acute water balance changes (Sands & Layton, 2009).

The second level—vascular—includes the countercurrent exchanger in the vasa recta, which minimizes but cannot completely prevent osmolyte washout by blood flow (Layton & Layton, 2005; Pallone et al., 2000, 2003). This level is critical for maintaining balance between oxygen and nutrient delivery to the medulla (requiring adequate blood flow) and osmotic gradient preservation (requiring washout minimization) (Pallone et al., 2003). Vasa recta blood flow regulation is accomplished by local vasoactive factors, particularly prostaglandins (PGE_2 and PGI_2, produced by RMIC and having vasodilatory effect) (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991; Pallone et al., 2003), nitric oxide, and endothelin; this regulation allows adaptation of perfusion to metabolic needs and kidney functional state (Pallone et al., 2003). The vascular level has no direct hormonal regulation by vasopressin (although high ADH concentrations can cause vasoconstriction through V1 receptors, but this is not

physiologically relevant at normal hormone concentrations) (Robben et al., 2006), but it closely interacts with epithelial and matrix levels through hemodynamic and osmotic connections (Layton & Layton, 2005; Pallone et al., 2003).

The third level—matrix—includes a dynamic osmolyte reservoir in the medullary interstitial matrix, where hyaluronic acid plays a central role (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968; Zhai et al., 2006). In the basal state (water diuresis, low vasopressin levels), the matrix contains high-molecular-weight HA (over 1 million daltons) (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992), which creates a viscous gel (Comper & Laurent, 1978), immobilizes Na^+ and urea through electrostatic interactions and volume exclusion effect (Comper & Laurent, 1978; Laurent, 1964; Maroudas, 1968), and provides structural support to the interstitium (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991). During vasopressin stimulation, hyaluronidase is activated (most likely HYAL2) through a cAMP-dependent mechanism (details of which require elucidation) (Cobbin & Dicker, 1962; Hoffert et al., 2006; Rowen & Law, 1981; Stern & Jedrzejas, 2006), leading to depolymerization of high-molecular-weight HA into medium and low molecular mass fragments (Cowman et al., 2015; Law & Rowen, 1981; Laurent & Fraser, 1992). Consequences are release of immobilized osmolytes (increasing effective interstitial osmolarity by 50-100 mOsm/kg) (Law & Rowen, 1981; Maroudas, 1968; Rowen & Law, 1981), viscosity reduction (facilitating diffusion and transport) (Comper & Laurent, 1978), decreased volume exclusion (increasing countercurrent multiplication efficiency) (Comper & Laurent, 1978; Layton & Layton, 2005), and creation of a buffering system that stabilizes the osmotic gradient at variable blood flow (Layton & Layton, 2005; Pallone et al., 2003; Rowen & Law, 1981). The matrix level contribution is approximately 60-65% of total concentrating response (Rowen & Law, 1981), and temporal dynamics are slower (hours) (Cobbin & Dicker, 1962; Law & Rowen, 1981), ensuring maintenance and stabilization of response during prolong ed antidiuresis (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981).

Critically important is the synergism among the three levels (Layton & Layton, 2005; Pallone et al., 2003; Rowen & Law, 1981; Sands & Layton, 2009). The epithelial level creates the basic osmotic gradient and provides water permeability (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995), but its effectiveness is limited by gradient stability in the interstitium (Layton & Layton, 2005). The vascular level provides metabolic needs but creates constant threat of osmolyte washout (Pallone et al., 2003). The matrix level stabilizes the gradient, protecting osmolytes from washout (Comper & Laurent, 1978; Law & Rowen, 1981; Maroudas, 1968; Rowen & Law, 1981) and increasing their effective concentration during vasopressin stimulation (Cobbin & Dicker, 1962; Law & Rowen, 1981). Dysfunction of any level can be partially compensated by others, explaining phenotypic variability in genetic defects (Arthus et al., 2000; Bichet, 2006): a patient with AVPR2 mutation (epithelial level defect) (Arthus et al., 2000; Robben et al., 2006) may have relatively preserved concentrating capacity if the matrix level functions optimally (high HA content, high basal hyaluronidase activity) (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), whereas another patient with the same mutation may have severe manifestations if the matrix level is also impaired (Arthus et al., 2000; Eddy, 1996; Kaissling & Le Hir, 2008).

This three-dimensional model has significant explanatory power for phenomena difficult to reconcile with traditional paradigm (Kuhn & Ryffel, 1942; Sands & Layton, 2009; Wirz et al., 1951). Osmotic gradient stability at high blood flow is explained by HA matrix buffering function (Comper & Laurent, 1978; Law & Rowen, 1981; Layton & Layton, 2005; Maroudas, 1968; Pallone et al., 2003; Rowen & Law, 1981). Pleiotropic effects of vasopressin (GAG content changes, appearance of hyaluronidase activity in urine) are explained by dual hormone action on epithelial and matrix levels through a single signaling cascade (cAMP) (Cobbin & Dicker, 1962; Hoffert et al., 2006; Law & Rowen, 1981; Nielsen et al., 1995; Robben et al., 2006; Rowen & Law, 1981). Phenotypic variability in genetic defects is explained by individual differences in matrix level function, which modulate consequences of epithelial defects (Arthus et al., 2000; Cowman et al., 2015; Garantziotis & Savani, 2019). Age-related decline in concentrating capacity is explained not only by decreased aquaporin expression and nephron number (Kortenoeven & Fenton, 2014; Lindeman et al., 1960; Sands & Layton, 2009) but also by impaired HA metabolism (Garantziotis & Savani, 2019; Reed et al., 1989), RMIC loss (Kaissling & Le Hir, 2008; Mutsaers et al., 2015), and interstitial fibrosis (Eddy, 1996; Kaissling & Le Hir, 2008). Early concentrating impairment in CKD is explained by interstitial pathology (fibrosis, inflammation, altered matrix composition) (Eddy, 1996; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008), which may precede significant epithelial function decline (Eddy, 1996; Sands & Layton, 2009).

The model also generates new predictions that can be tested experimentally and clinically (Kortenoeven & Fenton, 2014; Schuh et al., 2018). It predicts that modulation of HA metabolism can affect concentrating capacity independently of aquaporin status (Cowman et al., 2015; Garantziotis & Savani, 2019; Kortenoeven & Fenton, 2014; Rowen & Law, 1981): hyaluronidase inhibitors should worsen vasopressin response (Rowen & Law, 1981; Stern & Jedrzejas, 2006), while activators (if they can be identified) should enhance response or even partially compensate for epithelial level defects (Bichet, 2006; Cobbin & Dicker, 1962; Robben et al., 2006). It predicts that biomarkers of matrix function (HA fragments, hyaluronidase activity) should correlate with concentrating capacity and have prognostic value (Cowman et al., 2015; Garantziotis & Savani, 2019; Tammi et al., 2002). It predicts that genetic polymorphisms of HAS and HYAL should be associated with variability of

concentrating capacity in the population (Garantziotis & Savani, 2019; Tammi et al., 2002) and modulate phenotype in monogenic defects of the vasopressin-aquaporin system (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005). It predicts that interventions aimed at protecting or restoring RMIC function and normalizing extracellular matrix composition may have therapeutic potential in CKD and other conditions with impaired urine concentration (Eddy, 1996; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008).

CONCLUSIONS AND PERSPECTIVES

The concept of the renal medullary interstitial matrix as a dynamic reservoir of osmolytes, modulated by antidiuretic hormone through hyaluronidase activation (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), represents a paradigm shift in nephrology—a transition from a two-dimensional model (epithelium-vessels) (Kuhn & Ryffel, 1942; Pallone et al., 2003; Sands & Layton, 2009; Wirz et al., 1951) to a three-dimensional integrative model that includes the matrix as an active functional player rather than merely a passive structural scaffold (Comper & Laurent, 1978; Kaissling & Le Hir, 2008; Law & Rowen, 1981; Lemley & Kriz, 1991; Maroudas, 1968; Zhai et al., 2006). This concept is based on forgotten but elegant experiments from the 1960s-1980s that demonstrated a causal relationship between hyaluronidase activity and urine concentration (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), and is consistent with modern understanding of molecular properties of hyaluronic acid (Comper & Laurent, 1978; Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent, 1964; Laurent & Fraser, 1992; Maroudas, 1968), its distribution in the kidney (Law & Rowen, 1981; Zhai et al., 2006), and biology of renal medullary interstitial cells (Kaissling & Le Hir, 2008; Lemley & Kriz, 1991).

The dual action model of antidiuretic hormone postulates that vasopressin activates two parallel synergistic mechanisms through a single signaling cascade (V2R→cAMP) (Hoffert et al., 2006; Robben et al., 2006): the epithelial mechanism (PKA→AQP2 phosphorylation→trafficking→increased water permeability) (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995), which provides rapid response and contributes approximately 35-40% to the total effect (Rowen & Law, 1981), and the matrix mechanism (cAMP→hyaluronidase activation→HA depolymerization→release of immobilized osmolytes) (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981; Stern & Jedrzejas, 2006), which provides stabilization and enhancement of response and contributes approximately 60-65% to the total effect (Rowen & Law, 1981). This model explains osmotic gradient stability at high blood flow (Layton & Layton, 2005; Pallone et al., 2003; Rowen & Law, 1981), pleiotropic effects of vasopressin (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), phenotypic variability in genetic defects (Arthus et al., 2000; Bichet, 2006; Cowman et al., 2015; Garantziotis & Savani, 2019), and early concentrating impairment in chronic kidney disease (Eddy, 1996; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008).

A critical caveat is that most experimental evidence for the matrix mechanism comes from studies conducted several decades ago using methods now considered limited (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981), and that molecular details of cAMP-dependent hyaluronidase activation remain hypothetical (Hoffert et al., 2006; Stern & Jedrzejas, 2006). Therefore, validation of this concept using modern molecular, genetic, imaging, and clinical methods (Hoffert et al., 2006; Kortenoeven & Fenton, 2014; Schuh et al., 2018) is absolutely necessary before it can be confidently integrated into clinical practice. We have proposed a comprehensive validation research program that includes in vivo HA dynamics visualization (Schuh et al., 2018; Zhai et al., 2006), genetic modulation of HA metabolism (Garantziotis & Savani, 2019; Kortenoeven & Fenton, 2014; Stern & Jedrzejas, 2006; Tammi et al., 2002), interstitial microanalysis (Layton & Layton, 2005; Layton et al., 2015; Maroudas, 1968), elucidation of molecular mechanisms (Hoffert et al., 2006; Stern & Jedrzejas, 2006; Yasui et al., 1997), mathematical modeling (Layton & Layton, 2005; Layton et al., 2015), clinical biomarker studies (Arthus et al., 2000; Bichet, 2006; Cowman et al., 2015; Garantziotis & Savani, 2019), and comparative physiology (Garantziotis & Savani, 2019; Sands & Layton, 2009).

If this hypothesis proves valid, it will have profound implications not only for understanding fundamental renal physiology (Kuhn & Ryffel, 1942; Sands & Layton, 2009; Wirz et al., 1951) but also for clinical nephrology (Arthus et al., 2000; Bichet, 2006; Eddy, 1996; Fujiwara & Bichet, 2005; Robben et al., 2006). Biomarkers of interstitial matrix function (HA fragments of different molecular masses, hyaluronidase activity) (Cowman et al., 2015; Garantziotis & Savani, 2019; Tammi et al., 2002) may become tools for diagnosis, prediction of therapy response, and monitoring of disease progression (Arthus et al., 2000; Bichet, 2006; Eddy, 1996). Genotyping for polymorphisms of HA metabolism genes (Garantziotis & Savani, 2019; Tammi et al., 2002) may allow patient stratification and therapy personalization (Arthus et al., 2000; Bichet, 2006). Modulators of HA metabolism (Garantziotis & Savani, 2019; Stern & Jedrzejas, 2006) may become a new class of therapeutic agents for enhancing desmopressin effect in patients with partial response (Arthus et al., 2000; Bichet, 2006) or even for partial compensation of epithelial transport defects (Robben et al., 2006). Interventions aimed at protecting RMIC and normalizing extracellular matrix composition (Eddy, 1996; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008; Mutsaers et al., 2015) may slow chronic kidney disease progression (Eddy, 1996; Kaissling & Le Hir, 2008).

More broadly, this concept illustrates the importance of an integrative approach in biomedical sciences—an approach that is not limited to one level of organization (molecular, cellular, tissue) but attempts to understand how different levels interact and integrate to create complex physiological function (Hoffert et al., 2006; Kaissling & Le Hir, 2008; Layton & Layton, 2005; Sands & Layton, 2009). It also reminds us of the value of classical experimental works: observations by Cobbin and Dicker (Cobbin & Dicker, 1962), Law and Rowen (Law & Rowen, 1981), Rowen and Law (Rowen & Law, 1981) were made decades ago, but their significance can be fully appreciated only now, when we have the conceptual framework (Comper & Laurent, 1978; Garantziotis & Savani, 2019; Maroudas, 1968) and technological tools for their interpretation and validation (Hoffert et al., 2006; Kortenoeven & Fenton, 2014; Schuh et al., 2018). Science progresses not only through new discoveries (Agre et al., 1993; Knepper & Nielsen, 2004; Preston et al., 1992) but also through rethinking old data in light of new concepts (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981).

The path from intriguing hypothesis to established scientific fact is long and requires skepticism, rigor, and persistence (Baethge et al., 2019; Guyatt et al., 2011; Hooijmans et al., 2014; Page et al., 2021; Shea et al., 2017; Sterne et al., 2019). We invite the scientific community to critically evaluate the presented arguments, conduct the proposed experiments (Hoffert et al., 2006; Kortenoeven & Fenton, 2014; Layton & Layton, 2005; Schuh et al., 2018), and contribute to elucidating the role of the extracellular matrix in urine concentration (Comper & Laurent, 1978; Garantziotis & Savani, 2019; Kaissling & Le Hir, 2008; Law & Rowen, 1981; Maroudas, 1968; Rowen & Law, 1981; Zhai et al., 2006). Regardless of whether the final verdict is confirmation or refutation of our model, the very process of systematic investigation of this question will inevitably deepen our understanding of renal physiology (Sands & Layton, 2009) and open new horizons for fundamental and clinical research (Arthus et al., 2000; Bichet, 2006; Eddy, 1996; Garantziotis & Savani, 2019; Kortenoeven & Fenton, 2014).

KEY CONCLUSIONS ON HYPOTHESES

(1) Dual Action of ADH: Antidiuretic hormone acts through two complementary and synergistic mechanisms (Cobbin & Dicker, 1962; Hoffert et al., 2006; Law & Rowen, 1981; Nielsen et al., 1995; Rowen & Law, 1981)—epithelial (V2R → cAMP → PKA → AQP2 trafficking → increased water permeability, contribution 35-40%, rapid response 5-60 min) (Brown, 2003; Hoffert et al., 2006; Nielsen et al., 1995; Robben et al., 2006; Rowen & Law, 1981) and matrix (V2R → cAMP → hyaluronidase activation → HA depolymerization → release of immobilized osmolytes → increased effective osmolarity, contribution 60-65%, slower response 15 min - 4 hours) (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981; Stern & Jedrzejas, 2006). Both mechanisms are necessary for optimal urine concentration (Rowen & Law, 1981; Sands & Layton, 2009).

(2) Matrix Reservoir Function: Hyaluronic acid of the medullary interstitial matrix (concentration 50-100 μg/g, inner medulla/cortex gradient = 3-5:1) (Law & Rowen, 1981; Zhai et al., 2006) functions as a dynamic osmolyte reservoir, immobilizing Na^+ and urea (20-40% of total amount under basal conditions) through electrostatic interactions (polyanionic character of HA, -1 charge per disaccharide) (Maroudas, 1968) and volume exclusion effect (high-molecular-weight HA $>10^6$ Da creates steric constraints) (Comper & Laurent, 1978; Laurent, 1964). This creates a buffer reservoir that protects the osmotic gradient from washout by vasa recta blood flow (Layton & Layton, 2005; Pallone et al., 2003; Rowen & Law, 1981), ensuring stability under variable hemodynamic conditions (Layton & Layton, 2005; Pallone et al., 2003).

(3) Experimental Evidence: Classical studies from the 1960s-1980s provide convincing evidence of hyaluronidase's functional role in urine concentration (Cobbin & Dicker, 1962; Law & Rowen, 1981; Rowen & Law, 1981). The key experiment by Rowen and Law (1981) with antiserum against hyaluronidase showed that immunoneutralization of the enzyme blocks GAG degradation (by 60-70%) and suppresses the concentrating response to ADH (by 60-65%), providing direct evidence of causal relationship (Rowen & Law, 1981). These findings are consistent with the polyanionic properties of HA (Maroudas, 1968) and its distribution in the kidney (Law & Rowen, 1981; Zhai et al., 2006).

(4) Clinical Relevance: Interstitial matrix dysfunction may underlie phenotypic variability in urine concentrating disorders (Arthus et al., 2000; Bichet, 2006; Cowman et al., 2015; Garantziotis & Savani, 2019). In diabetes insipidus (central and nephrogenic), individual differences in HA metabolism may explain different symptom severity and desmopressin therapy response in patients with similar genetic defects (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005; Robben et al., 2006). In aging, impaired HA turnover (Garantziotis & Savani, 2019; Reed et al., 1989), RMIC loss (Kaissling & Le Hir, 2008; Mutsaers et al., 2015), and accumulation of pro-inflammatory HA fragments (Garantziotis & Savani, 2019) contribute to decreased concentrating capacity and increased dehydration risk (Lindeman et al., 1960; Sands & Layton, 2009). In CKD, medullary fibrosis (Eddy, 1996; Kaissling & Le Hir, 2008), altered GAG composition (Eddy, 1996; Kaissling & Le Hir, 2008; Law & Rowen, 1981), and RMIC loss (Kaissling & Le Hir, 2008; Mutsaers et al., 2015) lead to early concentrating dysfunction preceding GFR decline (Eddy, 1996; Sands & Layton, 2009).

(5) Biomarkers: HA fragments in urine and serum (especially molecular mass distribution: high-molecular-weight $>10^6$ Da, medium-molecular-weight 10^4-10^5 Da, low-molecular-weight $<10^4$ Da) (Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent & Fraser, 1992) and urinary hyaluronidase activity (Cobbin &

Dicker, 1962; Stern & Jedrzejas, 2006) are potential biomarkers of medullary interstitial matrix function. Validation of these biomarkers in clinical studies (Arthus et al., 2000; Bichet, 2006; Cowman et al., 2015; Garantziotis & Savani, 2019) may allow patient stratification for personalized therapy of water balance disorders (Arthus et al., 2000; Bichet, 2006; Fujiwara & Bichet, 2005).

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ACKNOWLEDGMENTS

The authors express deep gratitude to colleagues from the Ukrainian Research Institute of Transport Medicine (Odesa), Nicolaus Copernicus University (Toruń), and Bogomolets National Medical University (Kyiv) for valuable discussions, constructive criticism, and constant support during preparation of this manuscript.

We are also grateful to numerous researchers whose work in renal physiology (Kuhn & Ryffel, 1942; Layton & Layton, 2005; Pallone et al., 2003; Sands & Layton, 2009; Wirz et al., 1951), molecular biology of aquaporins (Agre et al., 1993; Hoffert et al., 2006; Knepper & Nielsen, 2004; Nielsen et al., 1995; Preston et al., 1992), glycosaminoglycan biochemistry (Comper & Laurent, 1978; Cowman et al., 2015; Garantziotis & Savani, 2019; Laurent & Fraser, 1992; Maroudas, 1968; Stern & Jedrzejas, 2006; Tammi et al., 2002), and clinical nephrology (Arthus et al., 2000; Bichet, 2006; Eddy, 1996; Fujiwara & Bichet, 2005; Kaissling & Le Hir, 2008; Robben et al., 2006) created the intellectual landscape in which integration of classical and contemporary concepts became possible.

CONFLICT OF INTEREST

The authors declare no financial or personal conflict of interest related to publication of this article. None of the authors has financial connections with companies manufacturing or developing diagnostic or therapeutic products relevant to the review topic.

FUNDING

This study received no specific financial support from governmental, commercial, or non-profit organizations. The work was performed within the framework of planned scientific research of the institutions where the authors work.

AUTHOR CONTRIBUTIONS

In accordance with authorship criteria of the International Committee of Medical Journal Editors (ICMJE), all authors made substantial intellectual contributions to this work:

Anatoliy I. Gozhenko (conceptualization 40%, literature review 35%, hypothesis development 40%, writing original draft of sections 1, 5-7, 11-12 constitutes 40%, overall project coordination 100%)

Walery Zukow (critical analysis of concepts 30%, methodological expertise 40%, editing and improvement of sections 2-4 constitutes 30%, development of validation research program 25%)

Olena A. Gozhenko (systematic literature search 30%, publication screening 40%, data extraction and systematization 50%, writing and editing sections 7-8 constitutes 30%, reference formatting and final manuscript preparation 80%)

Dmytro D. Ivanov (clinical implications analysis 70%, pediatric nephrology expertise 80%, phenotypic variability analysis 40%, biomarker concept development 40%, translational research design 50%)

All authors critically reviewed the intellectual content of the manuscript, provided important comments and suggestions, approved the final version for publication, and agree to be accountable for all aspects of the work, including ensuring accuracy and integrity of any part of it.