Novel Kidney Injury Biomarkers in Healthy Young Adults

by

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Abstract:

Chronic kidney disease (CKD) is a condition in which gradual kidney damage impairs its ability to filter blood. CKD has varying levels of severity and tends to become more common, and worsen, with advanced age. If left untreated, CKD can progress to kidney failure and early cardiovascular disease. About 37 million American adults are estimated to have CKD and most (~90%) are undiagnosed. Advanced CKD is more common in non-Hispanic Black adults than in non-Hispanic White adults or non-Hispanic Asian adults. Hypertension and health behaviors influence susceptibility to CKD. For example, high dietary salt is linked to future CKD. Due to the racial disparities and lifestyle factors that contribute to CKD, there is a growing interest in kidney injury biomarkers that are predictive of future CKD. As part of this thesis project, we sought to 1) determine whether high dietary salt increases kidney injury biomarkers in healthy young adults; and 2) we also sought to determine whether racial disparities play a role in kidney injury biomarkers within a college sample of healthy young Black and White adults.

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Chapter I Renal Physiology

A. Background - Organization of Urinary System

- ***Renal corpuscle (glomerulus + Bowman's capsule)***

The renal corpuscle is the blood filtering structure of the nephron that contains the glomerulus and Bowman's capsule.[1] The corpuscle is fed by an efferent arteriole carrying blood from the systemic circulation. The blood enters the glomerulus via the endothelial lining that drains into an efferent arteriole. The glomerulus is made of an endothelial lining containing fenestrated capillaries and a basement membrane. The fenestrated capillaries allow for diffusion of smaller particles out of blood.[2] The basement membrane of the glomerulus is where filtration occurs.[1] The basement membrane contains 3 important layers that increase the efficiency of filtration. The lamina densa is a dense region of type IV collagen and laminin that prevents larger particles from flowing into the filtrate.[1,3] The lamina densa is covered on both sides by the lamina rara interna and externa. The lamina rara interna is the region facing the endothelial lining while the lamina rara externa faces podocytes of the Bowman's capsule.[1] Both regions are concentrated with heparin sulfate, a negatively charged region, that acts to repel negative charges from being pulled into the filtrate.[1,3]

Further filtration occurs at the Bowman's capsule that contains a visceral and parietal layer. The visceral layer consists of podocytes that assist in this filtration process. The podocytes contain a "diaphragm slit" that permits the passage of particles smaller than 30 nm.[4] Each podocyte is connected via nephrin, a protein that further prevents the movement of particles larger than 7-9 nm.[4] Proteins that are able to make it through these

various barriers are drained into the proximal convoluted tubule. The parietal layer of the Bowman's capsule contains mesangial cells that provide three important functions: (1) the cells phagocytize large particles trapped in the nephron, (2) control blood flow through the afferent and efferent arterioles, and (3) are found in the gap junction with juxtaglomerular cells that respond to changes in blood pressure.[1]

- ***Proximal Convoluted Tubule***

The proximal convoluted tubule **(PCT)** is the first structure of the kidney tubule that immediately drains the renal corpuscle. Located in the renal cortex, reabsorption of most metabolites back into the bloodstream occurs here.[1] The structure is lined by simple cuboidal epithelial cells that contain an apical side that contains microvilli to assist the movement of molecules across the channel of the tubular lumen.[5] These cells contain many mitochondria to provide energy for transportation.

Nearly 100% of glucose, amino acids **(AA)** and vitamins, 90% of bicarbonate, 65% of Na+ and water, 60% of Calcium **(Ca^{2+})**, 50-55% of potassium **(K^+)** and chloride **(Cl^-)** reabsorption occurs here. Reabsorption occurs via passive and active transport with the PCT.[1] Passive diffusion of Na+ allows for secondary active transport of different molecules. Na+/K+ ATPase allows for movement of K^+ into the bloodstream against its concentration gradient.[1] This also occurs with glucose and amino acids via secondary active transport.

Carbon dioxide **(CO_2)** moves into the PCT to bind with water and interact with a carbonic anhydrase enzyme to form carbonic acid. Carbonic acid disassociates into

bicarbonate and H^+ protons.[6] This reaction allows for movement of H^+ ions into the filtrate and moving bicarbonate back into the bloodstream.[6]

Ca^{2+}, K^+, and magnesium **(Mg^{2+})** are reabsorbed via paracellular transport which involves them moving through tight junctions between cells.[1] Lipids also are also able to diffuse across the phospholipid bilayer to be reabsorbed into the bloodstream. Small proteins such as insulin and hemoglobin that may make their way into the filtrate are reabsorbed via endocytic receptors.[1] The proteins are then combined with lysosomes to be broken down into their AA constituents and moved into the bloodstream via fusion of vesicles.[1] Lastly, movement of phosphates requires an intracellular pathway involving an Na^+ and hydrogen phosphate **(HPO_4)** cotransporter. Parathyroid hormone binds to its receptor to activate a G-coupled receptor that activates the adenylate cyclase to produce cyclic AMP **(cAMP)** from ATP and generate protein kinase A **(PKA)**, which subsequently phosphorylates the cotransporter to inactivate it causing HPO_4 secretion.[1]

Tubular secretion into the filtrate in the PCT requires active transport. In a metabolic acidosis condition, glutamine within the tubular cells is deaminated and acidified to form ammonium **(NH_4^+)**. Ammonium is then oxidized to form 2 bicarbonate ions that are reabsorbed to increase blood pH. Cl- ions are then brought back into the cell to maintain the electronegativity. Any excess $NH4^+$ is secreted into the filtrate. Na+/H+ transporter allow for the movement of H+ ions into the filtrate and bicarbonate into bloodstream. Any excess drugs within the bloodstream also require ATP to be secreted into the filtrate.

- ***Loop of Henle***

The Loop of Henle **(LOH)** is the structure that succeeds the PCT, it extends into the adrenal medulla and is responsible for water conservation and concentration gradient of

the medulla. About 15% of water reabsorption and 25% of Na^+ reabsorption occurs here.[1] The thin descending LOH receives isotonic filtrate from the PCT. It is lined with simple cuboidal epithelium like the PCT.[5] The segment is only permeable to water and maintains an increasingly hypertonic filtrate as it goes down by allowing the movement of water in the medullary interstitial space via aquaporin-1 channels.[1] The turn in structure becomes the thin ascending LOH which is only permeable to ions. The thin ascending limb is composed of simple squamous epithelium.[5] The structure continues into the thick ascending LOH which contains $Na^+/K^+/Cl^-$ cotransporters that move the ions from the filtrate into the medullary interstitial space.[1] Similar to the descending limb, it is composed of simple cuboidal epithelium.[5] The ascending LOH creates an increasingly hypotonic filtrate. Some K+ ions are pushed back into lumen of the ascending limb causing depolarization of the apical side. This depolarization forces paracellular movement of Mg^{2+} and Ca^{2+} into the bloodstream.[1]

The countercurrent multiplier mechanism explains how the movement of solute and water allow for reabsorption and secretion of molecules in the LOH. The vasa recta, a peritubular capillary within the adrenal medulla that receives blood supply from the efferent arteriole, is responsible for this countercurrent exchange.[7] The vasa recta is a key structure preventing rapid removal of Na+ and Cl- while providing oxygen to the tubular cells. Blood flows slowly through the structure allowing for adequate reabsorption or secretion. As blood flows down the vasa recta, ions such as Na+ and Cl- are reabsorbed into the bloodstream while water is excreted into the medulla via aquaporin-1 channels.[7] As the vasa recta ascends, ions are pushed into the medullary interstitial space while water moves into the vasa recta.

- ***Distal Convoluted Tubule***

The distal convoluted tubule **(DCT)** traverses back into the renal cortex, coming after the ascending LOH. More reabsorption into the peritubular capillaries occurs here and it is composed of simple cuboidal epithelium.[5] The DCT is generally impermeable to water and requires hormone interaction to allow for its movement.[1] The structure can be broken down into an “early” and “late” section.

The “early” DCT is where ~5% of Na^+ reabsorption occurs. Na+/K+ ATPase along the basolateral membrane as well as Na^+/Cl^- symporters on the luminal membrane are responsible for the movement of Na^+ from the filtrate.[1] In response to hypocalcemia, parathyroid hormone **(PTH)** from the parathyroid gland bind to receptors on cells in the DCT activating the adenylate cyclase enzyme.[1] The PKA generated stimulates the opening of Ca^{2+} calmodulin channels on the luminal membrane for reabsorption. Na^+/Ca^{2+} along the luminal membrane also pump more Ca^{2+} into the blood using secondary active transport. Active transport of Ca^{2+} against its gradient via Ca^{2+}/H^+ channels also assist in elevating blood calcium levels.

In the late “DCT”, principal cells respond to aldosterone when blood Na+ levels are low and/or K^+ levels are high. Principal cells are responsible for maintaining mineral and water balance.[1] Aldosterone is able to diffuse the phospholipid bilayer and activate transcription factors to express proteins. These proteins are channels that allow for active transport of Na+ and K+ (Na/K-ATPase) and passive movement of the ions. Antidiuretic hormone **(ADH)** can also stimulate reabsorption of water and Na^+ by creating aquaporin-2 channels on the luminal membrane.[1]

- ***The Collecting Duct***

The collecting duct **(CD)** is the final structure that extends into adrenal medulla where urine is collected from the DCT. The structure is similar to the DCT where its permeability to water is guided by hormone interaction.[1] The CD contains both principal cells and intercalated A&B cells. The intercalated A&B cells contain similar structures that act is response to different physiological situations.

Intercalated A cells are active during acidosis in effort to bring blood pH back up. Excess CO_2 from the blood enters into the cells and combine with water via the carbonic anhydrase enzyme to form carbonic acid. Carbonic acid is then broken down into bicarbonate and H^+ ions. Active transport of H^+ ions into the filtrate occur via K^+/H^+ channels. These ions combine with ammonia to form ammonium in the filtrate. Bicarbonate is then reabsorbed to increase blood pH and replaced in the filtrate by C^{l-} ions to maintain electronegativity.[1] Intercalated B cells work in the opposite direction by secreting bicarbonate into the filtrate and reabsorption of H^+ ions via the same active transport channels.

The CD can also mediate water reabsorption when plasma osmolality is high or in the presence of angiotensin II **(ANG II)**.[1] In response to either situation, ADH is released from the posterior pituitary and binds to receptors on principal cells.[1] This activates the adenylate cyclase mechanism producing an end product of PKA that activates vesicles containing aquaporin-2 channels to fuse to the luminal membrane. An influx of water flows into the principal cell and enters the bloodstream via aquaporin-3 and 4 receptors located on the basolateral membrane of the principal cells. This leads to an increase in blood volume, blood pressure and less osmotic plasma. Urea reabsorption in the last portion of

the CD is critical in concentrating urine and maintaining the adrenal medulla concentration gradient.[1] Through the process of urea recycling, urea moves into the medullary interstitial space into LOH to drive water reabsorption.[7]

B. Glomerular Filtration and Renal Blood Flow

- *Glomerular filtration rate*

The glomerular filtration rate **(GFR)** is the amount of plasma volume that moves through the glomerulus in a given minute. The average estimated volume is around 125 ml/min. The GFR is a component of net filtration pressure multiplied by the filtration coefficient[4]. Net filtration pressure is the differences between the forces pushing out into the filtrate minus the forces pulling in or reabsorbing into the blood.

The net filtration pressure gradient established between the afferent and efferent arterioles delivering the blood to the glomerulus, which tends to push the plasma filtrate from the capillaries of the glomerulus into the capsular space.[4] The glomerular hydrostatic force represents the mechanical pressure exerted on the fluid pressure from cardiac output and arterial stiffness (elastic distension and recoil).[1] The opposing forces to the glomerular hydrostatic force include (1) the blood colloid osmotic pressure and (2) the capsular hydrostatic pressure. The blood colloid osmotic pressure represents the osmotic force (water concentration gradient) which tends to pull water from the plasma filtrate and back into the plasma in the glomerular capillaries. The blood colloid osmotic pressure results from the failure of most proteins to leave the plasma and move to the plasma filtrate, therefore, as water leaves, the proteins exert an increasing osmotic pressure on the water in the plasma filtrate.[4] The capsular hydrostatic pressure is the mechanical pressure exerted on the plasma filtrate by the elastic recoil of the glomerular capsule.

This force pushes water and dissolved solutes from the plasma filtrate back into the capillaries of the glomerulus. An average glomerular hydrostatic force is 55 mmHg, an average blood colloid osmotic pressure is 40 mmHg, and an average capsular hydrostatic pressure is 15 mmHg yielding a net outward pressure of 10 mmHg.[1]

The filtration coefficient is the second component of the GFR equation and is directly proportional to the surface area and permeability of the glomerulus.[1] An increase in glomerular surface area allows for increased filtration and vice versa.[4] An example of a reduction of surface area occurs in diabetic nephropathy where deposits and proteins cause the glomerulus to enlarge. In regard to permeability, the increase in permeability of the basement membrane can cause the membrane to become more porous, causing an increase of protein into the filtrate.[4]

C. Reabsorption and Secretion

Reabsorption and secretion are processes of the kidney where electrolytes, proteins and other foreign substances are moved between filtrate and the bloodstream. Both processes require either passive or active transport via ATP to allow non-permeable substances to cross the kidney tubule's membrane.[1]

Reabsorption is the process of bringing molecules back into the circulatory system and bloodstream through the use of the PCT, DCT, LOH, and the early portion of the CD. Beginning at the proximal tubule, reabsorption of water, ions and glucose is necessary to keep osmolality at ~300 mOsm. This process is called tubular reabsorption which requires the use of Na^+/K^+ ATP pump to allow for Na^+ ions to leave the filtrate for reabsorption. As a result, the kidney tubule Na^+ concentration decreases. Secondary active transport allows Na^+ ions to move passively down in ion gradient across the Na^+/glucose channel

and bring back glucose, lactate and amino acid molecules against the gradient and into the bloodstream.[1] These three molecules are nearly entirely reabsorbed. Bicarbonate is created through the reaction of CO_2 and water via the carbonic anhydrase enzyme to be reabsorbed. Water reabsorption is obligatory in the PCT as the molecule follows Na^+ into the peritubular capillaries.

The descending limb of the LOH is permeable to water only, so only water is reabsorbed here. The protein aquaporin I is present on the descending limb to assist with the diffusion of water. The filtrate that passes through the descending limb is hypertonic (up to 1200 mOsm) and encounters the ascending loop which is only permeable to solutes. $Na^+/K^+/2Cl^-$ cotransporters on the ascending loop brings metabolites into tubule cells where Na^+ and Cl^- are pumped into the interstitial space of the renal medulla. The vasa recta is a set of peritubular capillaries that run parallel to the Loop of Henle to supply and maintain that the renal medulla hypertonicity. Mg^{2+} and Ca^{2+} are reabsorbed through paracellular transport due to K^+ ions causing depolarization within lumen.

The “early” DCT contains Na^+/K^+ and specialized symporters that allow for Na^+ reabsorption. When blood Ca^{2+} levels are low, PTH is secreted from the parathyroid gland and uses the adenylate cyclase pathway to produce protein PKA to reabsorb Ca^{2+} back into the blood. The “late” DCT contains principal cells that respond to aldosterone which is able to pass through the basolateral lipid bilayer. Proteins are activated that allow for Na^+/K^+ pump to move Na^+ towards the blood and K^+ towards urine. ADH can be present to open aquaporins of the DCT to increase the reabsorption of water.

D. Salt and water balance

- ***Renin angiotensin aldosterone system***

The renin-angiotensin-aldosterone system **(RAAS)** is a hormone system of the kidneys that regulates blood pressure and fluid balance. In response to a reduction in blood pressure, renin is released from the juxtaglomerular **(JG)** cells of kidney vasculature.[1] Renin release is also stimulated by macula densa cells when Na^+ concentration is low in the "late" DCT or in response to increase sympathetic activity via JG b-1 receptors. Renin circulates in the bloodstream until it encounters angiotensinogen, a hormone produced in the liver. The enzyme cleaves angiotensinogen to form angiotensin I **(ANG-I)** which is further converted to ANG-II by angiotensin converting enzyme **(ACE)** endothelial cells in the lungs and kidneys.[1] ANG-II increases blood pressure through vasoconstriction of smooth muscle and increasing stroke volume through water retention; the hormone stimulates the pituitary gland to produce ADH and the adrenal glands of the kidneys to produce aldosterone.[1] Cells in the adrenal cortex produce cholesterol that are key in aldosterone production. High K^+ levels in the "late" DCT and CD can also stimulate aldosterone production.

Aldosterone stimulates an increase in Na^+/K^+ pump activity while creating channels on the principal cells for increased perfusion of Na^+ and K^+.[1] The K^+ channels on the apical surface of the cell push K^+ ions towards filtrate. This action further increases Na/K^+ activity due to the decreasing level of K^+ ions in the principal cell. The Na^+ channels are on the apical surface of the principal cells to bring in Na^+ ions to an area of lower concentration in the membrane. These Na^+ ions are eventually pumped into the peritubular capillary

towards the bloodstream. Water follows the Na^+ ions into the bloodstream as well causing an increase in stroke volume.

Aldosterone also facilitates intercalated a-cells of the CD to respond to acidosis in the blood. These cells produce CO_2 and water which are converted to bicarbonate and H^+ by way of the carbonic anhydrase enzyme.[1] Cl^- ions are transported into the cell to allow bicarbonate into the bloodstream to interact with the high [H^+]. The interaction creates CO_2 and water, increasing stroke volume and ventilation. Aldosterone increases production of Na^+/H^+ gradient and K^+/H^+ active transport for moving solutes between filtrate and urine.

- ***Transport of Ions (Na^+, K^+, Cl^-)***

Na^+ transportation and regulation are key factors in extracellular fluid **(ECF)** homeostasis as water follows it. The element accounts for a large portion of the ECF and filtrate osmolality. The largest percentage of Na+ reabsorption occurs at the PCT followed by the LOH, DT, and CD. Na^+ reabsorption in the renal tubular occurs from two pathways: transcellular and paracellular. Transcellular reabsorption involves movement of Na^+ and Cl^- passively into the apical membrane and the removal of Na+ from the basolateral movement through Na^+-K^+ pumps. Paracellular reabsorption involves Na^+ and Cl^- movement through tight junctions between cells. The transepithelial electrochemical gradient is responsible for Na^+ transport. If the driving force is positive then it favors Na^+ reabsorption, if negative then favors secretion. Paracellular pathways benefit from segments leakiness of Na^+ ions which provides the voltage for current flow between the basolateral and apical membranes.

- ***Transport of Urea, Inorganic and Organic Solutes***

Apart from transport of ions, urea and glucose, and proteins also play a role in fluid balance. Urea is a nitrogenous waste product generated by the kidney during protein catabolism. It is freely filtered at the glomerulus, reabsorbed in the PCT and CD, and secreted in the thin LOH.[1] Urea is used to concentrate urine. Urea excretion is directly proportional to urine flow rate. Glucose is freely filtered in the glomerulus and reabsorbed primarily in the proximal tubule. Glucose reabsorption requires transcellular active transport through Na/glucose cotransporters.[1] The basolateral Na-K pump help maintain Na+ concentration to drive process. Glucose is almost never present in urine of healthy adults.

Large proteins are generally blocked from filtrate by the basement membrane. Albumin specifically is reabsorbed about 96-99% out of filtrate.[1] Tubular and glomerular injury are certain cases were proteinuria is found. Excess protein is reabsorbed by endocytosis in the PCT.[1]

E. Clinical measures of renal function and novel kidney injury markers

Clinical assessments of renal function are imperative for medical practice. Accurate and reliable measurement of GFR is critical for determining the efficacy of interventions to slow or reverse progressive renal disease, and for making clinical decisions regarding patient care.[8] Moreover, GFR measures are important parameters used to assess renal function in research studies seeking to identify the prognostic utility of assessments of renal function.[8,9] Measurements of GFR are generally accepted to reflect the number of normally functioning glomeruli in the kidney. Notably, there are several techniques used

to measure GFR in humans. Many of these approaches quantify urinary excretion of a labeled tracers that are ideally excreted solely via glomerular filtration, meaning they are neither secreted, nor reabsorbed by tubules. With constant infusion rates, or a constant endogenous production rate, the steady-state blood concentration of these substances can then be determined by GFR.[9] Non-steady-state methods may also be used to determine GFR based on plasma disappearance rate of the tracer following a bolus injection of the tracer.[9]

Exogenously infused molecules include as inulin, iothalamate, iohexol, and radioactive markers (^{125}I-iothalamate, ^{99m}Tc-DTPA, ^{51}Cr-EDTA).[9,10] Clearance measures of these molecules are considered a gold standard for assessing GFR in both clinical and animal studies. However, the utility of exogenous clearance determinations in the clinic is limited by high expense, the significant expertise needed, the requirement of intravenous administration, as well as concomitant timed urine sample collection.[10-12] Therefore, measuring endogenous molecules is an attractive alternative for measuring GFR and kidney injury on a more routine basis. We will discuss endogenous measures below.

- ***Clinical measures kidney function and acute kidney injury***

There are several measures commonly used clinically to assess kidney function and acute kidney injury. Below, I will describe the common clinical measures of GFR followed by a discussion of acute kidney injury **(AKI)** markers. Creatinine is a 113 dalton amino acid derivative generated through catabolism of creatine in muscle and filtered by the kidneys to be excreted in urine via glomerular filtration.[13] Once seen as a gold standard

to measure renal function, creatinine is now recognized as being a highly variable measure and having several limitations in determining estimated GFR.[14] For example, creatinine is secreted by proximal tubules and filtered by glomerulus causing an overestimation of GFR by creatinine clearance.[12,14,15] Moreover, serum creatinine values vary when considering muscle mass, age, gender and ethnicity. Men can have a higher GFR at the same creatinine levels due to higher average muscle mass and creatinine production than women. Regarding race, Black individuals have a higher GFR at similar creatinine levels as others due to higher average muscle mass and creatinine production. Serum creatinine can be affected by diets that are rich in protein.[15]

Creatinine clearance can be measured from serum creatinine and creatinine excretion or estimated from serum creatinine alone using estimating equations.[9,13] Measurement of creatinine clearance requires collection of a timed urine sample (generally 24-hour sample). Multiple organizations such as the National Institute of Diabetes and Digestive and Kidney Diseases, National Kidney Foundation, and American Society of Nephrology also recommend estimating GFR **(eGFR)** from serum creatinine based on commonly used equations in clinical and research settings. Two of the most commonly used equations include the Modification of Diet in Renal Disease **(MDRD)** Study equation and the CKD-EPI equations.[16,17] The MDRD was developed in 1973 and uses serum creatinine in combination with age, sex, weight, or race to calculate eGFR, while the CKD-EPI equation was developed in 2009 to assess eGFR also using serum creatinine, age, sex, and race. Because the MDRD and CKD-EPI equations use variables such as age, sex, and race, individuals with similar serum creatine will have very different eGFR. As such, using serum creatinine, alone, is not a rigorous technique to assess renal

function. The use of serum creatinine alone is particularly problematic in the early stages of renal dysfunction because a rise in blood creatinine concentration is not observed until after significant loss of functioning nephrons.[15] Due to the limitations of using serum creatinine alone, a more rigorous assessment of GFR is using creatinine clearance via a serum sample and urine from a 24-hour urine collection.[18] Additional measures of kidney function apart from estimated GFR and creatinine clearance include measuring albumin, urea, and cystatin C.

Albumin is a protein source found in diet that aids the body in growth and tissue repair. When albumin levels are in a healthy range, fluid from swollen tissue will be able to move into blood more readily to be filtered. Only small amounts of albumin are excreted in the urine.[19] Albuminuria is increased excretion of urinary albumin and a marker of kidney damage.[20] Researchers use the ratio of albumin to creatinine in determining reduced GFR as it is more sensitive than looking at elevated albumin levels alone.[20] Albuminuria levels are classified as microalbuminuria (< 30-300 mg/g) and macroalbuminuria (< 300 mg/g). Urine albumin levels are considered a key marker in kidney injury and disease.[19]

Urea is a nitrogenous waste product in blood that comes from catabolized protein from the diet. It is comprised of two amino groups and a linked carbonyl group. Urea is created in the Urea Cycle when proteins are broken down into toxic ammonia and converted to non-toxic urea.[21] The kidneys adjust urea reabsorption and secretion as the filtrate passes through the tubule determines an important role for urea in the production of a maximally concentrated urine.[22] The protein is useful for determining estimated GFR. An increase in the ratio between serum and plasma urea shows an

imbalance between urea production in the liver and urea elimination by the kidneys. A limitation of urea is that it doesn't accurately represent reduced GFR. A prior investigations determined that plasma/serum urea concentrations were not altered until there was an approximately 50% reduction in GFR.[22]

Cystatin C is a non-glycosylated, 13.3-kDa protein belonging to cystatin protease inhibitors.[23] Cystatin C is produced by nucleated cells as a biomarker of inflammation and excreted during glomerular filtration. The protein is then completely reabsorbed in the tubules before being broken down in the proximal tubule.[23,24] In contrast to creatinine , an advantage to Cystatin C is that serum cystatin C concentration is unaffected by gender, age, race, protein intake, and muscle mass.[24] However, a limitation to the biomarker is that it is elevated in the presence of excess inflammation, and in elderly populations.[24] Some studies have shown the use of urinary cystatin C to be a possible early biomarker of AKI post-surgery and as a predictive measure of nephropathy.[24,25] Recent studies have clearly demonstrated that cystatin C is a better predictor of adverse events in the elderly serum creatinine or estimated GFR.[26]

AKI is common in hospitalized patients after traumatic events or years of kidney damage, and is associated with increased morbidity, mortality, and cost. Currently, AKI is diagnosed after symptoms already manifest. Some of the available diagnostic tests, including eGFR, urine microscopy, and proteinuria have limited ability to identify subclinical AKI.[27] Preclinical experimental studies and clinical studies have identified biomarkers that enable earlier recognition of AKI or even identify patients at risk of AKI.[27] Additionally, some of these biomarkers has shown promise as biomarkers associated with incident chronic kidney disease, which is a major cause of mortality and

morbidity. Below, I will discuss two of these novel AKI biomarkers; Neutrophil gelatinase-associated lipocalin and Kidney Injury Molecule-1.

Neutrophil gelatinase-associated lipocalin **(NGAL)** is a kidney injury biomarker protein of the lipocalin family. Human NGAL exists as a monomer, a 45-kDa homodimer and a 135-kDa heterodimer where it is conjugated to gelatinase and is specific to neutrophils.[28] The monomer is the gene product itself, which is very rapidly secreted from stimulated or damaged epithelial cells. The known functions of NGAL are related to its ability to bind iron–siderophore complexes and exert a bacteriostatic function of the innate immune system.[29] The iron–siderophore complexes are separated to prevent iron uptake by bacteria).[29] This function can also be co-opted to transport iron to the cytoplasm via catecholate–iron complexes where it activates or represses iron-responsive genes.[30]

Regarding site of production, NGAL is produced in the distal nephron and is upregulated in response to kidney injury.[31] NGAL is generated in the Loop of Henle and collecting duct and released in the urine. Plasma NGAL is produced systemically and is reabsorbed in the proximal tubule but can be found in urine if there is damage to the tubule.[31] Due to its many origins, NGAL can be detected easily in blood and urine and is upregulated in instances of acute kidney injury and chronic kidney disease **(CKD)**.[32] The classic outcome for determining AKI is a 50% increase in serum creatinine.[31] However, NGAL allows for quicker assessment of injury compared to serum creatinine or eGFR. In a prior clinical study using NGAL and serum creatinine as biomarkers, NGAL was able to detect and diagnose AKI with 2-6 hours of surgery compared to 1-3 days with serum

creatinine.[31] Therefore, the time course and sensitivity of NGAL enables the detection of AKI prior to, and without, changes in serum creatinine levels.

NGAL is versatile in determining varying kidney injuries and their outcomes. NGAL is sensitive gene to tubular cell stress and damage via the pathway triggered by sepsis.[30] With sepsis being the primary symptom for AKI, NGAL concentration can rise days before sepsis causes serum creatinine elevation reducing the need for dialysis. Elevations in the biomarker are used to predict incidence of death and dialysis in AKI, with large levels (<104 ng/ml) showing a 15% incidence rate.[30]

Kidney Injury Molecule-1 (KIM-1) is a type 1 transmembrane signal glycoprotein with an extracellular immunoglobulin-like domain topping a long mucin-like domain, produced on the apical surface of epithelial cells in the proximal tubule.[30] KIM-1 aids in recovery and tubular regeneration due to it acting as a phosphatidylserine receptor that mediates phagocytosis of apoptotic bodies and debris into renal epithelial cells.[28,33] The extracellular domain of KIM-1 is shed from the surface by a metalloproteinase-dependent process.[34] Shedding of the extracellular domain along with intrarenal KIM-1 mRNA and protein production cause an increase in KIM-1 urine concentration after AKI. KIM-1 concentration increases in response to ischemia in the kidneys.

One study demonstrated that KIM-1 was expressed in regenerated epithelial cells as an adhesion molecule that assists cell interaction postischemia injury.[34] With a normal range concentration in urine being less than 1 ng/ml, KIM-1 levels increase to 3-7 ng/ml within 6 hours of injury and remain elevated for 48 hours.[35] Upregulation of the KIM-1 limits inflammation during AKI by suppressing the Ga_{12} protein, which inhibits

phagocytosis of apoptotic cells, and blocks ROS-mediated injury pathways that lead to further tissue damage.[33] The biomarker is also useful for predicting AKI in surgery, possible nephrotoxicity or injury in preclinical studies of pharmaceuticals and detecting certain cancers.[35] Kidney tubular expression of KIM-1 is a result of damage and inflammation in the region. The biomarker's presence up to 3 hours after injury allows for a quicker assessment of injury than serum creatinine.

Chapter II Influence of high dietary salt on novel urinary biomarkers of kidney injury in healthy young adults

Introduction

The Dietary Guidelines for Americans and the American Heart Association have long-standing recommendations to limit sodium consumption to no more than 2,300 mg sodium per day [36]. However, most Americans continue to overconsume dietary sodium, and the average American adult consumes ~ 3,500 mg sodium per day [37,38]. Notably, high dietary sodium increases the risk of cardiovascular and kidney disease [39-41]. Given the damaging effects of excess dietary sodium on the kidneys, there is growing interest in sensitive biomarkers of kidney injury to identify individuals who may be at higher risk for developing future kidney disease with high salt consumption. Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) are established kidney tubule injury biomarkers [42-44] and have shown promise as biomarkers of acute kidney injury and chronic kidney disease in the general population [42,45-48]. Multiple rodent studies demonstrate that high dietary sodium increases urinary excretion of urinary NGAL [49-51] and in some cases KIM-1 [51]. A recent investigation in older adults also demonstrated that a modest reduction from habitual dietary sodium reduced urinary concentration of NGAL [52].

Blood pressure (BP) variability is associated with target organ damage [53-55], and preclinical data demonstrate that high dietary salt increases BP variability [56]. From a clinical standpoint, increased BP variability with salt loading could contribute to organ damage in high-flow-resistance organs such as the kidney. However, the limited human data on salt and BP variability is equivocal [57,58]. Cross sectional data indicate that salt intake is associated with BP variability in patients with hypertension [57]. In contrast, we recently demonstrated that ten days of controlled high salt feeding, comparing to low and recommended sodium, did not increase BP variability in healthy adults [58]. Additionally, in patients with hypertension, high salt intake is associated with high glomerular filtration rate (GFR; > 135 ml/min) [59], which is prognostic of future cardiovascular events [60]. In otherwise healthy adults there are data suggesting high salt does [61-63] and does not [63,64] influence GFR.

We recently demonstrated that high dietary salt loading led to signs of BP dysregulation and vascular dysfunction in a cohort of healthy young adults using a randomized, placebo-controlled, crossover study [65,66]. However, it is unclear if short-term changes in sodium intake influences kidney injury biomarkers, kidney function, and BP variability in healthy adults. Therefore, the primary purpose of this study was to determine whether short-term high dietary sodium increases urinary excretion of the kidney injury biomarkers NGAL and KIM-1 in healthy young adults using the same cohort. We hypothesized that short-term high dietary sodium would increase urinary excretion of NGAL and KIM-1. Because it is unclear whether high dietary salt elicits changes GFR in healthy adults, we assessed creatinine clearance, urinary cystatin C, and urinary urea as additional measures of kidney function, and we speculated GFR would be unaffected in

this cohort of young and recreationally active adults. Lastly, we sought to determine whether beat-to-beat BP variability was adversely influenced (i.e., increased) by salt manipulation in this cohort. The findings of this investigation will provide additional insight into the relation between dietary salt and future kidney disease by determining whether short-term high dietary sodium increases kidney injury biomarkers and increases BP variability, which could contribute to kidney injury, in healthy adults.

Methods

Study participants

All participants provided written and verbal consent prior to engaging in any study activities. The study protocol and procedures were approved by the Institutional Review Board of the University of Delaware; they conform to the provisions of the *Declaration of Helsinki*, and the trial was registered on clinicaltrials.gov (NCT03565653). The primary data reported here consisted of a post-hoc analysis using frozen urine and plasma samples. Additional details of the parent study, conducted from 2017 to 2019, have been provided in two prior publications [65,66].

After providing consent, study participants underwent a medical history screening. We measured body height (cm) and mass (kg) for calculation of the body mass index (BMI; kg/m^2). We measured seated BP via oscillometric assessment in triplicate following at least five minutes of seated rest (Dash 2000, GE medical systems). The average of the triplicate BP measures is reported here. Participants also completed the physical activity readiness questionnaire. Participant age ranged from 18 to 34 years, and all participants were recreationally active (self-reported exercise $\geq$ 3 days/week). Exclusion criteria included a history of hypertension, diagnosis of cardiovascular disease, cancer, type 1 or

type 2 diabetes, kidney dysfunction, current pregnancy, obesity (BMI > 30 kg/m^2), and current use of tobacco products (i.e., within preceding six months).

Sodium intervention

We randomized participants into a double-blind, placebo-controlled, crossover study. We instructed participants to consume a recommended sodium diet (2,300 mg per day) in each of two 10-day periods. We provided participants with instructions for interpreting nutrition labels and meeting sodium intake requirements. Participants also consumed unmarked capsules each day containing either salt (Morton® table salt (NaCl); 3,900 mg sodium per day) or a placebo (NOW Foods® dextrose). Total sodium intake was designed to be 6,200 mg/day during the high-salt condition and 2,300 mg/day during the dextrose condition. We randomized (https://www.randomizer.org/) the condition order and separated conditions by ≥ two weeks for male participants and by one month for female participants. All female participants were using oral hormonal contraceptives, and experimental visits occurred during the inactive week of oral contraceptives. Participants recorded their diet during the first intervention and were asked to match their diet during the second intervention via a copy of their initial diet log. We analyzed three days of diet records from a subset of participants including two weekdays and a weekend day using Nutrition Data System for Research (NDSR).

24-hour urine collection

Urine was collected during the final 24 hours of both interventions in a light-protected, sterile 3500-mL container. Participants were instructed to abstain from alcohol, caffeine, and exercise for the 24 hours prior to and during the 24-hour urine collection.

Participants returned the container at their experimental visits. We measured total urine volume, urine specific gravity (Goldberg Brix Refractometer, Reichert Technologies), urinary electrolyte concentrations (EasyElectrolyte Analyzer, Medica), and urinary osmolality (Advanced 3D3 Osmometer, Advanced Instruments) from a mixed aliquot of the 24-hour urine collection container. The urine flow rate was derived from urine volume and self-reported time the participant used the container (placebo: 1357 ± 135 minutes, salt: 1378 ± 192 minutes, $p = 0.616$). We also stored mixed aliquots from the 24-hour collection container in cryogenic tubes at –80° C.

Experimental visits

On the tenth day of each diet, participants reported to the Cardiovascular Physiology Laboratory at the University of Delaware for their experimental visits. Participants provided a spot urine sample for assessment of urine specific gravity (Goldberg Brix Refractometer, Reichert Technologies). Samples from female participants were tested using hormonal pregnancy tests (Moore Medical) to ensure that they were not pregnant. We measured body mass, body composition, and total body water via bioelectrical impedance (Tanita Body Composition Analyzer, Model TBF-300A; Arlington Heights, IL). Participants were instrumented for single-lead electrocardiography and oscillometric BP measurements at the upper arm (right arm; Dash 2000, GE medical systems) following ≥ 15 minutes of supine rest. Beat-to-beat BP was recorded via finger photoplethysmography (Finometer, Finapres Medical Systems) at the middle finger of the left hand, which was supported at the heart level. Systolic BP and diastolic BP were defined as the maximum and minimum values, respectively, from the arterial BP

waveform during each cardiac cycle. Mean arterial BP was calculated as the integral of the BP waveform [58,67].

Venipuncture and biochemical analysis

We obtained venous blood samples via venipuncture after ≥ 15 minutes of supine rest. To obtain plasma samples, we spun venous blood samples in K^+EDTA-treated vacutainers at 750 g for 15 minutes at 4° C (Allegra X-22R, Beckman Coulter), removed the plasma layer, re-spun plasma at 750 g for 15 minutes at 4° C, and carefully pipetted all plasma into cryogenic tubes to be stored at –80° C. As previously described [65,66], we also assessed serum (spray-coated silica vacutainer) electrolytes (EasyElectrolyte Analyzer, Medica), plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments), hemoglobin (Hb 201+, HemoCue), and hematocrit (Sure prep™ capillary tubes, Clay Adams spun in a microcentrifuge at 1,950 g for five minutes, Legend Micro 17, Thermo Sorvall). As previously described [68], we calculated the change in plasma volume (expressed as a percentage) using the following equation:

$$\text{Plasma volume (\%)} =$$

$$((100(Hb_{PLA}/Hb_{HS})x(1 - (Hct_{HS}/100))/((1 - Hct_{PLA}/100)) - 100)),$$

where *PLA* is the placebo condition, *HS* is the high-salt condition, Hb is hemoglobin, and Hct is hematocrit.

Assays for kidney function

We measured plasma NGAL and KIM-1 and urine NGAL, KIM-1, cystatin C, and urea concentrations in triplicate using enzyme-linked immunosorbent assays (ELISAs), and kinetic endpoints were read on a Biotek synergy H1 plate reader. We obtained the

ELISA kits to measure plasma and urine NGAL from Toronto BioScience (cat. 31050) [69]. We obtained the ELISA kits to measure plasma KIM-1 (DSKM100), and urine KIM-1 (DKM100) from R&D Systems. We obtained the ELISA kits to measure urine cystatin C (ab179883) and urea (ab83362) from Abcam. The intra-assay coefficients of variation were as follows: 3.1% for plasma NGAL and 4.6% for plasma KIM-1, 6.7% for urine NGAL, 4.4% for urine KIM-1, 4.3% for cystatin C, and 3.5% for urea. We performed all of the assays using manufacturer-provided instructions, except for urine KIM-1, for which we loaded 200 µL (instead of 50 µL) of sample because of low reported kit sensitivity [52] and calculated the respective concentration (i.e. [concentration]x(50 µL/200 µL)). Based on prior publications and manufacturer recommendations [30], we used the following dilutions for urine samples: 1:10 for NGAL, 1:200 for cystatin C, and 1:5000 for urea. For urinary KIM-1, samples from four participants were below the sensitivity of the ELISA; however, we ran additional quality control samples (QC24; R&D Systems) to ensure validity of the values from all remaining samples. All the values reported here for the other ELISAs were above the minimum detectable sensitivity for each of the respective kits. In addition to the undetectable KIM-1 concentration, urinary NGAL is missing from one participant. However, sample sizes are indicated in each graph. We calculated NGAL and KIM-1 excretion rates (Figures 1B and 2B) as follows: concentration of the assayed sample (ng/mL) x flow rate (ml/min), resulting in ng/min units.

We used frozen urine and serum samples to measure creatinine via kinetic modification of the Jaffe procedure; the measurements were performed at Christiana Care, Newark, DE; Lab Services. As previously described [59], we assessed creatinine clearance, both adjusted and unadjusted for body surface area, as a measure of GFR

using urine and serum creatinine concentration and urine flow rate. Body surface area was calculated as follows:

$$\frac{\sqrt{height(cm)\ x\ mass(kg)}}{3600}$$

We also calculated estimated GFR using serum creatinine (along with sex, age, and race of the participants) using the CKD-EPI equation [70]. Based on prior publications, we defined a "high glomerular filtration rate" as > 135 mL/min, [59,71]. We also calculated the fractional excretion of sodium from sodium concentration in the serum and urine, and the change in serum and urinary creatinine concentration [59,69]. Creatinine clearance data is missing from two participants due the self-reported urine volume resulting in a creatine clearance greater than 1.5 × the interquartile range.

Blood pressure variability

We assessed beat-to-beat BP variability using the BP signal obtained via photoplethysmography, as previously described [58,67,72]. Briefly, participants were supine for > 15 minutes prior to testing, consistent with prior publications [58]. We calculated beat-to-beat BP variability over 10 min of quiet rest in the laboratory. We assessed BP variability using the standard deviation (SD) and the average real variability (ARV) index. The SD method has frequently been used for BP in the literature and is associated with cardiovascular morbidity and mortality [73]. However, the ARV of BP has greater prognostic value,[74] and is seen as a more precise representation of BP variability than the SD. Specifically, rather than measuring total variance within an array of BP measures, the ARV expresses the absolute difference of consecutive BP measurements and is calculated using the following formula [74]:

$$ARV = \frac{1}{\sum w} * \sum_{k=1}^{N-1} w * |BP_{k+1} - BP_k|,$$

where *N* is the number of BP readings, *k* indicates the order of the measurements, and *w* is the time interval between BP_k and BP_{k+1}. Thus, we assessed BP variability using both SD and ARV.

Statistical analysis

The statistical approaches reported here were informed by recent guidelines for statistical reporting of cardiovascular research [75]. We tested the effects of salt pills on blood and urine measures using paired, two-tailed *t*-tests when data were normally distributed. When data were not normally distributed, we used Wilcoxon matched-pairs signed rank tests. We tested plasma volume change using a one-sample *t*-test. Statistical significance was set at $p < 0.05$. We also calculated effect size for select variables to provide further context when interpreting data. Specifically, we calculated Cohen's *d* as the ratio of mean difference between salt and placebo conditions divided by the pooled standard deviation, (Cohen's $d = (M_{salt} - M_{placebo})/SD_{pooled}$). Statistical analyses were completed using SPSS (IBM, version 26.0) and Prism (GraphPad, version 9). We report data as mean ± SD throughout.

Results

Participant characteristics including age, sex, body mass index, BP and estimated GFR are reported in Table 1. Participants were non-obese and had normal to elevated BP.

Responses to interventions. We present the effects of the capsule intervention on physical and biochemical characteristics in Table 2. Notably, average urinary sodium excretion during the placebo condition was ~ 130 mmol/day, or ~ 2,990 mg/day. However, ten days of salt capsules significantly increased average urinary sodium excretion to ~ 287 mmol/day, or ~ 6600 mg/day, and plasma volume was higher by 7.2% after 10 days of the salt intervention as compared to after 10 days of the placebo intervention (Table 2). Total body mass and serum chloride were higher after 10 days of the salt intervention as compared to after 10 days of the placebo intervention. In contrast, systolic, mean, and diastolic BP, plasma osmolality, and serum sodium and potassium were not different between conditions. Systolic, mean, and diastolic BP ARV were also not different between conditions (Table 2). As compared to after 10 days of the placebo intervention, the salt loading intervention resulted in a modestly, yet significantly greater SD of systolic BP (placebo = 5.6 ± 2.2 vs. salt loading = 6.5 ± 1.9 mmHg, p = 0.035). However, the SD of mean BP (placebo = 4.5 ± 1.6 vs. salt loading = 4.9 ± 1.4 mmHg, p = 0.186) and diastolic BP (placebo = 3.9 ± 1.4 vs. salt loading = 4.4 ± 1.2 mmHg, p = 0.091) were not different between conditions.

NGAL. Plasma NGAL concentration was not different between conditions (placebo = 8.0 ± 4.4 vs. salt loading = 8.3 ± 4.2 ng/mL, Figure 1A). Salt loading significantly increased urinary NGAL excretion rate (placebo = 59.8 ± 44.4 vs. salt loading = 80.8 ± 49.5 ng/min, Figure 1B). Apart from being significantly increased, the effect size for the key outcome measure, urine NGAL excretion rate (Cohen's *d* = 0.46), was moderate. However, urinary NGAL concentration indexed to creatinine was not different between conditions (placebo = 0.730 ±0 .635 vs. salt loading = 0.741 ± 0.490 ng/mg Figure 1C).

KIM-1. Plasma (placebo = 21.5 ± 13.5 vs. salt loading = 21.4 ± 11.2 ng/mL, Figure 2A) and was not different between conditions. Urinary KIM-1 concentration indexed to the flow rate (placebo = 0.85 ± 1.03 vs. salt loading = 0.58 ± 0.64 ng/min, Figure 2B) and urinary KIM-1 concentration indexed to creatinine (placebo = 0.0076 ± 0.0069 vs. salt loading = 0.0052 ± 0.0053 ng/mg, Figure 2C) were also not different between conditions.

Kidney function. Indices of kidney function are presented in Table 3. Compared to the placebo intervention, salt loading resulted in greater creatinine clearance, both adjusted and unadjusted for body surface area, and greater fractional excretion of sodium. Compared to the placebo intervention, salt loading resulted in a higher incidence of high GFR when assessed via the creatinine clearance *unadjusted* for body surface area. However, when assessed via the creatinine clearance *adjusted* for body surface area, there was no difference in the incidence of high GFR. Urinary cystatin C excretion was greater after salt loading compared to the placebo intervention. Urinary urea excretion was not different between conditions (Table 3). Lastly, urine volume was modestly but significantly greater after 10 days of salt loading compared to the placebo intervention (placebo = 1763 ± 812 vs. salt loading = 1969 ± 925 mL/day, p = 0.047).

Regarding the participant's self-reported nutrition data from their diet records, there was no difference in reported sodium intake between conditions (placebo = 2506 ± 779 vs. salt loading = 2752 ± 554 mg/day, p = 0.493). Based on the 24-hour urinary sodium excretion following 10 days of placebo (2990 mg/day) and the reported sodium intake on the placebo intervention (2506 mg/day) it appears the participants' underreported dietary sodium by ~16 percent.

Discussion

The key findings of this investigation were that short-term dietary salt loading increases urinary excretion of the kidney injury biomarker NGAL in healthy young adults. While NGAL was not increased when indexed to creatinine, this finding could be attributable to salt loading also eliciting increased in creatinine excretion (compared to placebo) in our cohort. A large body of literature demonstrates that high dietary salt is associated with future development of chronic kidney disease [41] and adverse health outcomes associated with kidney disease, such as initiation of dialysis and mortality [76]. Our investigation provides unique insight, demonstrating that even ten days of high dietary salt elicits an increase in urinary NGAL excretion, an established clinical biomarker of kidney tubular injury.

Our observation that dietary salt results in increased urinary NGAL excretion is also consistent with another recent investigation that reported that five weeks of dietary salt restriction resulted in a reduction in urinary NGAL concentration in 13 healthy middle-aged and older adults with moderately elevated BP [52]. In contrast to NGAL, we found that urinary excretion of kidney injury biomarker KIM-1 was not elevated after 10 days of high dietary salt. While there are rodent data demonstrating that high dietary salt results in increased KIM-1 excretion, it took approximately four months for hypertensive rats being fed a high-salt diet to exhibit elevated urinary excretion of KIM-1 [51]. In contrast, normotensive rats fed a high-salt diet do not exhibit elevated urinary excretion of KIM-1 even after several weeks to months of high dietary salt [49,50]. Taken together, these findings indicate that NGAL may be a more sensitive marker of kidney injury and that even in more at-risk populations (e.g., hypertension) it may take longer-term high dietary salt to increase KIM-1 excretion. In the only other human study that we are aware of that

investigated the influence of high dietary salt on KIM-1, the investigators were unable to detect KIM-1 in urine samples in all but one of the participants [52]. Thus, there is a need for future studies to assess KIM-1 after longer-term habitual or experimental high dietary salt consumption, and in higher-risk populations.

Interestingly, in our cohort, high dietary salt increased urinary NGAL even though participants did not experience different resting BP between diets. We also assessed BP variability, because greater variability could also contribute to aberrant patterns of blood flow to the kidneys. BP variability is associated with target organ damage [53-55], and given our hypothesis that salt loading would elicit kidney injury, we sought to determine whether BP variability was also increased. Changes in BP variability are regulated by redundant homeostatic mechanisms, including the baroreceptor reflex, sympathetic tone, the renin–aldosterone–angiotensin system, and bioavailability of nitric oxide, all of which can be affected by dietary sodium [77,78]. While SD of systolic BP was significantly increased with salt loading in the present investigation, systolic BP ARV was not, neither were measures of diastolic and mean BP variability. It is worth noting that in addition to the SD of systolic BP being significantly increased with salt loading, the effect size was also moderately large (Cohen's *d* = 0.44). However, given that five of our six measures of BP variability were not changed with salt and that the ARV is seen as a more precise measure of beat-to-beat BP variability (as well as a stronger prognostic indicator) [74], it appears that BP variability was only minimally influenced by salt loading. Moreover, using regression analysis, we determined that the SD of systolic BP was not correlated with NGAL excretion (R^2 = 0.002, p = 0.781).

In contrast to our findings in healthy humans, high dietary salt elevates 24-hour BP variability in salt-resistant rodents with normotensive BP [56]. Additionally, 24-hour urinary sodium excretion is positively correlated with 24-hour BP variability in patients with hypertension [57]. In contrast, we recently demonstrated that beat-to-beat and 24-hour BP variability were not influenced by salt manipulation in a cohort of healthy adults who were largely salt resistant and who had normotensive BP [58]. There are caveats to our largely null BP variability findings. For example, controlled salt loading interventions may result in increased BP variability in higher-risk populations (e.g., older adults, patients with hypertension) and in cohorts with a proportion of individuals with salt-sensitive BP. Moreover, in this study and our other recent investigation [58] were relatively short (i.e., 10 days). While speculative it could be that high dietary salt administered over longer periods of time elicits increased BP variability.

Given that our cohort was composed of healthy young adults who did not have salt-sensitive BP (i.e., a change of mean arterial BP of > 5 mmHg), we did not anticipate that high dietary salt would elicit a large increase in creatinine clearance [63,64]. However, there are prior investigations demonstrating that short-term (i.e., one to several weeks) high dietary salt increased GFR in generally healthy adults [61-63]. Importantly, a sustained elevation in GFR is an independent predictor of adverse CV events [60]. While the incidence of high GFR when adjusted for body surface area was not significantly increased with salt loading in the present cohort, the incidence of GFR doubled when unadjusted for body surface area. Additionally, there was a significant increase in creatinine clearance, irrespective of body surface area adjustment. The changes in creatinine clearance exhibited by our cohort suggest that there may have been an increase in kidney perfusion

pressure with salt loading, although this remains speculative. Underscoring the clinical relevance of our GFR findings, a recent cross-sectional investigation also determined that 24-hour urine sodium excretion is an independent predictor of GFR in adults with hypertension and that habitual high salt consumption is associated with a higher prevalence of high GFR [59].

The aforementioned study on sodium and GFR in patients with hypertension also found that high salt intake was associated with protein catabolism from endogenous muscle or excess exogenous dietary protein, as demonstrated by higher urea clearance [59]. Interestingly, prior translational studies using controlled feeding in male cosmonauts and rodent models have also demonstrated that salt loading promotes protein catabolism [79,80]. The previous publications included speculation that high salt elicits protein catabolism to provide osmotically active urea which conserves body water as a counter-regulatory mechanism to increased sodium excretion. However, it has traditionally been thought that high dietary salt is associated with transiently increased serum sodium and osmolality, which subsequently stimulates thirst, leading to greater fluid consumption and increased plasma volume [81,82].

In the present investigation, we did not find a difference in urea excretion between conditions, and participants did exhibit higher urine volume with salt loading, but they also exhibited increased plasma volume, body mass, and fractional sodium excretion, all of which indicate at least some degree water retention. Similarly, a recent secondary analysis of the DASH-Sodium trial also demonstrated that four weeks of high dietary salt was not associated with changes in urea excretion [81]. While we did not assess thirst in our study, thirst was increased with higher dietary sodium in the DASH-Sodium trial and

there was a slight increase in body weight despite energy intake not being different between diets [81]. Thus, our current findings and the DASH-Sodium findings indicate that high salt does not lead to protein catabolism or the utilization of urea as a fluid-conservation mechanism. One potential reason for the discrepancy regarding high salt and protein catabolism between the trials could be that the cosmonaut study and the cross-sectional study involved long-term high dietary sodium (several months to years), whereas our trial and the DASH-Sodium trial were short term (i.e., ≤ four weeks per condition).

In addition to creatinine clearance and urea, we also measured cystatin C to assess kidney function. Cystatin C is a 13-kDa cysteine protease inhibitor and is produced by all nucleated cells at a constant rate [83]. In healthy participants, cystatin C is nearly freely filtered by the glomeruli and almost entirely reabsorbed and catabolized in the proximal tubules [83]. Therefore, we speculated that cystatin C would likely not change with dietary sodium, as it is influenced to a lesser extent by factors such as age, biological sex, and muscle mass than creatinine is [84,85]. However, cystatin C excretion was significantly increased (large effect size, Cohen's d = 0.92). Thus, we speculate that the increase in cystatin C may be the result of kidney injury and reduced reabsorption in the proximal tubule, which has been associated with progression of nephropathies [84,86].

Our study does have important limitations. All participants were given the same sodium load whereas normalizing sodium to body mass or caloric intake (i.e. controlling for sodium density) may provide additional translational value. We also intended for our participants to consume 2,300 mg of sodium per day on the placebo arm of the study. However, their urinary sodium excretion while on the placebo (~ 2990 mg) indicates that

they consumed closer to habitual levels of dietary salt. These findings illustrate the difficulty the general population experiences in reducing their dietary sodium intake, which is likely a reflection of the fact that the vast majority (~ 70%) of the sodium Americans consume is already in foods they typically eat, such as packaged food items and restaurant meals [36]. It also remains to be determined whether habitual sodium intake is linked cross-sectionally with kidney injury markers. However, a prior study indicating that even modestly reducing habitual sodium reduces urine NGAL concentration in older adults suggests that this may be the case [52]. The existing preclinical data [49-51] and the human data discussed here highlight the need for future investigations in large, diverse cohorts to further examine the role of dietary salt and kidney injury markers as well as underlying mechanisms.

Nonetheless, important strengths of our study include that 24-hour urine collection is the gold standard for assessment of sodium intake, as it is estimated to account for ≥ 90% of dietary sodium intake [87]. Additionally, we used a within participant, crossover design to reduce variance. Given the significant different between salt loading and placebo interventions and the moderate effect size we are confident that salt loading did substantially increase NGAL excretion in this cohort of healthy adults despite the relatively modest sample size. Lastly, given that the participants in this investigation were generally healthy and young, our findings suggest that it is important for Americans to take preventative action to reduce sodium consumption from a cardiorenal perspective. The results may be even more pronounced with longer-term high sodium intake and in older adults, those with hypertension, and individuals with salt-sensitive BP. In conclusion,

dietary salt loading elicited high GFR and increased urinary excretion of the kidney injury biomarker marker NGAL after short-term high sodium intake in healthy young adults.

Chapter III Potential racial differences in novel urinary biomarkers of kidney injury in healthy young adults

Introduction

Non-Hispanic Black adults are at elevated risk of developing cardiovascular disease [88-90] and end-stage kidney disease[91,92] compared to White adults. There is further evidence indicating divergent development of kidney dysfunction among racial lines.[93-95] For example, within the Multi-Ethnic Study of Atherosclerosis (MESA), compared to White Adults, Black adults had a higher risk of kidney function decline as assessed by estimated glomerular filtration rate (eGFR) using kidney serum creatinine and Cystatin C.[94] Both Black and Hispanic adults had the highest rates of incident chronic kidney disease (CKD) amongst others with eGFR > 90 ml/min per 1.73 m^2. In a separate study using the Racial Differences in Stroke (REGARDS) Cohort, stage 3 CKD, as defined by an eGFR <60 ml/min per 1.73 m^2 was slightly less prevalent among black than white patients.[95] However, at progressively higher stages of CKD (i.e., eGFR of <20 ml/min per 1.73 $m^{2)}$ Black patients were disproportionately represented, providing further evidence that kidney function declines at an accelerated rate in Black adults.[95]

Similar to advanced CKD and end-stage kidney disease, there are stark differences in acute kidney injury (AKI) in adulthood. In 2016, the rate of AKI

hospitalizations among older Medicare patients was 72 per 1000 patient-years in Black Americans and 45 per 1000 patient-years in White Americans.[96] A recent study focused on COVID-19 outcomes demonstrated that even after adjustment for confounders self-reported Black race was associated AKI prevalence in patients with COVID-19.[97] Importantly these observations do not explain the reasons behind the disparities in kidney disease and AKI prevalence. However, an investigation from the Atherosclerosis Risk in Communities (ARIC) study sought to address this knowledge gap.[98] The investigation found that there was a racial disparity, whereby Black adults had a prevalence of hospitalization for AKI compared to White adults. Annual family income, education level, and prevalence of health insurance were lower among Black adults as well. Moreover, accounting for differences in income and/or insurance by race attenuated the association while adjustment for cardiovascular risk factors and high-risk APOL1 variants did not.[98] Taken together, the findings discussed here illustrate that there are racial disparities in advanced kidney disease and AKI prevalence in middle-aged to older adults. There is a need for more research on potential disparities in younger adults and the underlying environmental and social determinants that contribute to the disparities in kidney health.

An investigation using the Bogalusa Heart Study dataset found that eGFR was high in Black compared to White middle-aged adults. Serial blood pressure measurements that started in childhood were predictive of eGFR (i.e., a negative association) in Black but not White adults.[99] The findings of this study indicate that blood pressure control throughout adulthood is important for regulating kidney function in Black adults. Regarding AKI, the rate of AKI hospitalizations among Medicare patients older than 22 years old was 5.6 per 1000 patient-years in Black Americans and 4 per 1000

patient-years in White Americans.[96] Thus, the prevalence was low for both races, however, it is unclear if there are racial differences in subclinical AKI in generally healthy Black and White adults. Therefore, the purpose of this investigation was to determine whether there are racial differences in kidney injury biomarkers Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). Importantly, Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) are established kidney tubule injury biomarkers [42-44] and have shown promise as biomarkers of acute kidney injury and chronic kidney disease in the general population.[42,45-48] We obtained 24-hour samples from participants to measure NGAL and KIM-1, and also measured creatine clearance (i.e., using serum and urine creatinine) to assess GFR. We hypothesized there may be a racial difference in kidney injury biomarkers. Our eventual goal is to increase the sample and determine whether social determinants such as socioeconomic status and experiences of racism are associated with in kidney injury biomarkers.

Methods

Recruitment

Participants were undergraduate college students at a large four-year university in the Southeastern United States. The student body was 86% White, 4% Black, 3% Hispanic, 2% Asian, and 5% other or multiracial at the time of recruitment. Using student records to identify demographic characteristics, equal numbers of first- and second-year Black and White students were invited to participate. White students who were first generation (did not have a parent who had graduated from a four-year college) were over-sampled to reduce potential confounding by socioeconomic status. Inclusion criteria was age

between 18 and 25, and race of Black/African American or White in student records. In total, 468 Black and 608 White students (33%first generation in both groups) were sent information (pamphlet and letter) about the study via mail (to their local and permanent addresses) and emails (to their university email address). The study was described as seeking to understanding factors in students' lives that influence their sleep and health. Responding students were screened for sleep disorders during a lab visit using established measures of apnea, narcolepsy, restless leg syndrome, or severe insomnia.[100-103] No differences in response rates were observed by age or first-generation college student status. Exclusion criteria were (1) reporting a sleep disorder, (2) screening above threshold values on sleep disorder screening measures, or (3) having a serious medical condition that interferes with sleep or precludes wearing an actigraph. The study protocol involved a lab visit preceded by a 3-day sleep actigraphy and sleep diary assessment. For this particular study, students were invited for an additional study visit at that entailed measures of vascular function preceded by a 24-hour urine collection to assess sodium intake and measures of kidney function. All study procedures complied with ethical guidelines for human subject's research and received approval from an institutional review board.

Venipuncture and biochemical analysis

We obtained venous blood samples via venipuncture after ≥ 10 minutes of supine rest. To obtain serum samples, we spun venous blood samples in silicone-treated vacutainers at 1500 g for 10 minutes at 4° C (Sorvall ST8R, ThermoFisher), removed the serum layer, and re-spun if necessary due to clotting. We carefully pipetted all plasma into cryogenic tubes to be stored at –80° C. As previously described [65,66], we assessed

plasma (spray-coated silica vacutainer) electrolytes (Smartlyte Electrolyte Analyzer), plasma osmolality (Advanced 3D3 Osmometer, Advanced Instruments), hemoglobin (Hb 201+, HemoCue), and hematocrit (Sure prep™ capillary tubes, Clay Adams spun in a microcentrifuge at 1,950 g for five minutes, Legend Micro 17, Thermo Sorvall).

24-hour urine collection

Urine was collected for 24 hours before the study visit in a light-protected, sterile 3500-mL container. Participants were instructed to abstain from alcohol, caffeine, and exercise for the 24 hours prior to and during the 24-hour urine collection. Participants returned the container at their experimental visits. We measured total urine volume, urine specific gravity (Refractometer), urinary electrolyte concentrations (Electrolyte Analyzer), and urinary osmolality (Osmometer, Advanced Instruments) from a mixed aliquot of the 24-hour urine collection container. The urine flow rate was derived from urine volume and self-reported time the participant used the container (-). We also stored mixed aliquots from the 24-hour collection container in cryogenic tubes at –80° C.

Assays for kidney function

We measured urine NGAL concentration in triplicate using enzyme-linked immunosorbent assays (ELISAs), and kinetic endpoints were read on a Biotek synergy H1 plate reader. We obtained the ELISA kits to measure urine NGAL from Toronto BioScience (cat. 31050) [69]. In the future the laboratory plans to obtain ELISA kits to measure urine KIM-1 (DKM100) from R&D Systems. The intra-assay coefficients of variation was 4.21% for urine NGAL. Based on prior publications and manufacturer recommendations [30], we used a 1:10 dilution for NGAL. Sample sizes are indicated in

each graph. We calculated NGAL excretion rate as follows: concentration of the assayed sample (ng/mL) x flow rate (ml/min), resulting in ng/min units.

We used frozen urine and serum samples to measure creatinine via kinetic modification of the Jaffe procedure; the measurements were performed at the University of Alabama at Birmingham Bioanalytical Core (Clinical Biochemical Genetics Laboratory). As previously described [59], we assessed creatinine clearance, both adjusted and unadjusted for body surface area, as a measure of GFR using urine and serum creatinine concentration and urine flow rate. Body surface area was calculated as follows:

$$\frac{\sqrt{height(cm)\ x\ mass(kg)}}{3600}$$

We also calculated estimated GFR using serum creatinine (along with sex, age, and race of the participants) using the CKD-EPI equation [70]. Creatinine clearance data is missing from the participants due the self-reported urine time of less than 18 hours or 1080 minutes.

Statistical analysis

The statistical approaches reported here were informed by recent guidelines for statistical reporting of cardiovascular research [75]. We assessed racial differences on measures of interest using paired, two-tailed *t*-tests when data were normally distributed. When data were not normally distributed, we used Mann-Whitney U tests. Statistical significance was set at $p < 0.05$. We also calculated effect size for select variables to provide further context when interpreting data. Specifically, we calculated Cohen's *d* as the ratio of mean difference between salt and placebo conditions divided by the pooled standard deviation, (Cohen's $d = (M_{salt} - M_{placebo})/SD_{pooled}$). Statistical analyses were

completed using SPSS (IBM, version 26.0) and Prism (GraphPad, version 9). We report data as mean ± SD throughout. In the future we plan to assess differences in kidney injury markers among racial groups using regression models that also incorporate important covariates such as sleep, physical activity, vitamin D status, socioeconomic status, and neighborhood similar to prior publications led by Dr. Thomas Fuller-Rowell and colleagues.[104-106]

Results

Physical and Biochemical Measures. Our analytical sample consisted of 57% White and 43% Black with 51% being female. In total there were 10 black females, 6 black males, 9 white females and 12 white males. Race differences of physical and biochemical measures are shown in Table 4. Body mass ($p = 0.973$), height ($p = 0.062$) and BMI ($p = 0.125$) were not different between groups. Black participants had significantly higher systolic (110 ± 9 vs. 103 ± 8 mmHg, $d = 0.79$, $p = 0.023$) and diastolic ($d = .87$, $p = 0.013$) than White participants.

Kidney Function Measures. 24-hour urine volume (1237.2 ± 899.8 vs 2638.8 ± 1338.1 ml, $d = 1.20$, $p < .001$) and urine flow rate (0.8 ± 0.56 vs. 1.76 ± .0.91 ml/min, $d = 1.23$, $p < .001$) were greater amongst White participants compared to Black participants but 24-hour Na^+ excretion did not differ between groups ($p = 0.505$). Serum creatinine did not differ between Black and White participants ($p = 0.884$) but there was a significant difference in urine creatinine concentration (199.9 ± 115.1 vs. 97.0±76.6 mg/dl, $d = 1.08$, $p = 0.003$) with Black participants producing urine with a greater concentration. This was likely due to the large difference in urine volume such that Black participants produced

more concentrated urine. Despite the greater urine creatinine concentration in Black participants, creatinine clearance ($p = 0.966$) and eGFR ($p = 0.887$) were not different between groups. See Figure 3 for a visual depiction of urine flow rate, creatinine clearance and eGFR

NGAL. Urinary NGAL concentration was significantly increased in Black participants when compared to White participants (36.4 ± 28.46 vs. 15.9 ± 19.34 ng/ml, $d = 0.87$, $p = 0.017$). However, when indexed to flow rate ($p = 0.374$) and creatinine ($p = 0.774$), there were no differences between groups. See Figure 4.

Discussion

The key findings of our investigation were that contrary to our hypothesis, young Black adults did not excrete more urinary NGAL than young White adults. Of these participants, there were also not differences in creatine clearance or estimated dietary sodium intake. Overall, the cohort was generally young and healthy. Additionally, the sample size is currently small ($n < 50$ total). As the laboratories continue to collect additional data the results of the investigation may evolve, and the conclusions may change. Additional experiments are needed to assess potential differences in KIM-1 as well. However, it could be that there are not racial differences in kidney injury biomarkers.

Overall, little is known about the prevalence of kidney injury amongst youth and young adults. Some studies have shown children with elevated BP have a larger prevalence of microalbuminuria, an indication of renal damage.[107,108] The Bogalusa heart study in Louisiana attempted to correlate childhood BP with future incidence of ESRD and determine whether there were racial differences. There was a higher incidence of

microalbuminuria in Black participants even those who fell within “normal” BP ranges.[109] Adult BP was negatively associated with adult eGFR compared to White participants, indicating higher prevalence of BP related renal damage but there were no significant associations between eGFR and childhood BP.[99] This may indicate that differences may not develop until midlife, further underscoring that evidence is needed to determine whether there are racial differences in renal function in healthy, young adults.

Regarding middle-aged and older adults, socioeconomic, genetic and environmental factors, such as racism have been proposed as possibilities for the increased prevalence of kidney disease faced by Black Americans. A prior study examined the associations of genetic African ancestry and socioeconomic status with kidney function cross-sectionally and longitudinally in community-dwelling sample of Black Americans.[110] Serum creatinine and cystatin C were used to assess eGFR. There was no association between genetic African ancestry and either measure of eGFR (creatinine and cystatin C) and African ancestry was not associated with longitudinal changes in serum creatinine. However, self-reported African-American race was strongly associated with increased risk for CKD progression. Moreover, low income was strongly associated with prevalent kidney dysfunction by cystatin C.[110] In contrast, a subsequent investigations examining participants the Coronary Artery Risk Development in Young Adults (CARDIA) using 42 ancestry informative markers found that African ancestry was independently associated with elevated creatinine among Black men, although not women.[111] Additionally, a graded increase in odds of elevated serum creatinine (i.e., reduced eGFR) was found among Black men as percentage of African ancestry increased.[111] Importantly the authors noted higher African ancestry may be a marker for

social factors not measured in the study such as individual poverty, neighborhood poverty, social cohesion, access to health care, access to healthy food due to neighborhood and finances, discrimination, stress, or other environmental exposures. For example, higher African ancestry is associated with darker skin color [112], and darker skin has been associated with more frequent encounters of racial discrimination.[113]

Our study results also show discrepancies in urine volume and hydration status between whites and blacks. White participants had a larger urine volume (2638 vs. 12327 ml, see Table 4) when compared to Black participants, indicating a larger water intake. Prior studies using National Health and Nutrition Examination Survey data have shown differences in hydration status between Black and White Americans adults and children.[114-117] Additionally, there is a recent pilot study demonstrating racial differences in hydration among college students.[118] The reasons for these differences are not entirely clear but socioeconomic factors may play a role. For example, perceptions of unsafe tap water among racial and ethnic minorities are well documented.[116,119,120] Interventions to improve access to clean water and improve education on hydration are warranted. We plan to assess hydration markers such as urine osmolality and specific gravity and look at dietary food and hydration logs to further determine any differences as the study continues.

In conclusion, additional data is needed for us to determine whether there may be racial differences in urinary excretion of kidney injury biomarkers. Specifically, our laboratory still needs to measure urinary KIM-1 in our cohort, and we need to continue to build upon the sample size of our cohort. As alluded to above, there are social determinants that likely contribute to racial disparities in advanced kidney disease

prevalence. Recent data indicate that there are racial differences in sleep health[104,121] in college students and that neighborhood disadvantage may contribute to sleep disparities.[104] There are also data in college students indicating racial disparities in BP variability which could contribute to pulsatile flow to the kidney if autoregulation of renal pressure is at all impaired.[122] These data informed our hypothesis that there may be racial disparities in kidney injury biomarkers in young adults, however, additional data is needed.

Tables

Table 1. Participant descriptive characteristics at screening. Data are presented as mean ± SD. Abbreviations: NHW = non-Hispanic White, BR = biracial, A = Asian, H = Hispanic, eGFR = estimated glomerular filtration rate; derived from CKD-EPI equation.

Participant Characteristics		
Variable	Mean ± SD	Range
Age (years)	24.2 ± 3.9	18–34
n (Female/Male)	20 (8/12)	–
Race/Ethnicity	13 NHW, 1 BR, 4A, 2H	–
Body Height (cm)	172.8 ± 9.8	152.4–185.4
Body Mass (kg)	69.4 ± 12.5	48.6–96.8
BMI (kg/m^2)	23.1 ± 2.6	18.4–28.9
Brachial Systolic BP (mmHg)	112 ± 10	86–136
Brachial Mean BP (mmHg)	80 ± 8	50–80
Brachial Diastolic BP (mmHg)	64 ± 8	64–93
eGFR (ml/min)	126 ± 27	87-176

Table 2. The effects of salt loading on blood pressure and biochemical measures, as mean ± SD. Abbreviations: ARV = average real variability, Na^+ = sodium, K^+ = potassium, Cl^- = chloride. ARV was derived from beat-to-beat blood BP readings.

	Placebo	Salt	*p* value
Body Mass (kg)	68.8 ± 12.8	69.2 ± 12.8	**0.042**
Brachial Systolic BP (mmHg)	108 ± 11	108 ± 8	0.902
Brachial Mean BP (mmHg)	77 ± 7	77 ± 6	0.830
Brachial Diastolic BP (mmHg)	61 ± 7	61 ± 6	0.914
Systolic BP ARV (mmHg)	0.99 ± 0.40	1.09 ± 0.44	0.140
Mean BP ARV (mmHg)	0.89 ± 0.29	0.95 ± 0.36	0.290
Diastolic BP ARV (mmHg)	0.99 ± 0.36	1.03 ± 0.41	0.553
Urine Na^+ Excretion (mmol/24hr)	130.3 ± 62.4	287.2 ± 72.0	**< 0.001**
Plasma Osm (mOsm/kgH_2O)	294 ± 5	294 ± 5	0.691
Serum Na^+ (mmol/L)	141.0 ± 1.8	141.4 ± 2.1	0.319
Serum K^+ (mmol/L)	4.1 ± 0.5	4.1 ± 0.5	0.505
Serum Cl^- (mmol/L)	104.8 ± 2.0	106.2 ± 1.9	**0.017**
Plasma Volume Change (%)	7.2 ± 11.3		**0.012**

Table 3. The effects of salt loading on measures of kidney function, as mean ± SD. Abbreviation Na^+ = sodium

	Placebo	Salt	*p* value
Urine Creatinine excretion (mg/day)	1240 ± 398	1598 ± 231	**< 0.001**

Serum Creatinine (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.552
Unadjusted Creatinine Clearance (mL/min)	110.5 ± 32.9	145.4 ± 23.9	**< 0.001**
High GFR (%)	(6/18) 33%	(12/18) 67%	**0.046**
Adjusted Creatinine Clearance (/1.73m^2)	105.6 ± 30.8	137.0 ± 23.7	**< 0.001**
High GFR (%)	(6/18) 33%	(10/18) 56%	0.180
Urinary Cystatin C excretion (mg/day)	0.15 ± 0.06	0.22 ± 0.09	**0.015**
Urea Excretion (g/day)	18.8 ± 13.4	19.9 ± 13.7	0.543
Fractional Excretion of Na^+ (FENa)	0.62% ± 0.39%	0.98% ± 0.29%	**< 0.001**

Table 4. Participants descriptive characteristics from screening and biochemical measures as mean ± SD. Abbreviation Na^+ = sodium

`	Black	White	p-value	Cohen's d
Sample Size	n = 16	n = 21	-	-
(Female/Male)	(10 F/6 M)	(9 F/12 M)	-	-
Age	0.0 ± 0.0	0.0 ± 0.0	p = 0.000	d = 0.00
Height (cm)	167.2 ± 7.2	172.9 ± 10.1	p = 0.062	*d = 0.64*
Mass (kg)	75.8 ± 13.5	76.0 ± 13.7	p = 0.973	d = 0.01
BMI (kg/m^2)	27.2 ± 4.7	25.0 ± 3.5	p = 0.125	*d = 0.52*
Brachial SBP (mmHg)	110 ± 9	103 ± 8	**p = 0.023**	*d = 0.79*
Brachial DBP (mmHg)	67 ± 9	60 ± 7	**p = 0.013**	**d = 0.87**
Urine Volume (ml)	1237.2 ± 899.8	2638.8 ± 1338.1	**p < 0.001**	**d = 1.20**
24hr Urine Na^+ Excretion	168.3 ± 117.0	143.9 ± 103.0	p = 0.505	d = 0.22
Urine Creatinine (mg/dl)	199.9 ± 115.1	97.0 ± 76.6	**p = 0.003**	**d = 1.08**
Serum Creatinine (mg/dl)	0.86 ± 0.33	0.85 ± 0.15	p = 0.884	d = 0.05

Figures

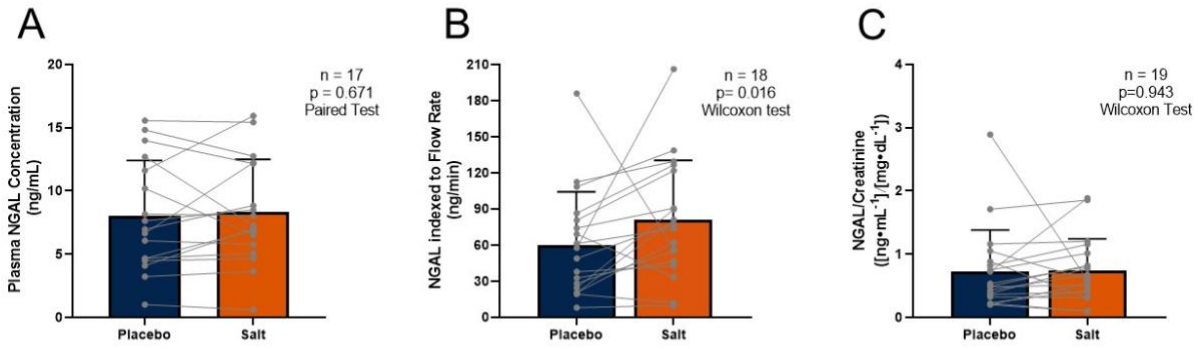

Figure 1. The effects of high dietary salt intake on NGAL. (A) Plasma NGAL was not affected by salt loading. (B) Urine NGAL indexed to urine flow rate were increased after 10 days of dietary salt loading. (C) NGAL indexed to creatinine concentration was not affected by salt loading. Sample sizes and statistical tests are reported in each graph. All data are presented as individual data points overlaid with mean ± SD.

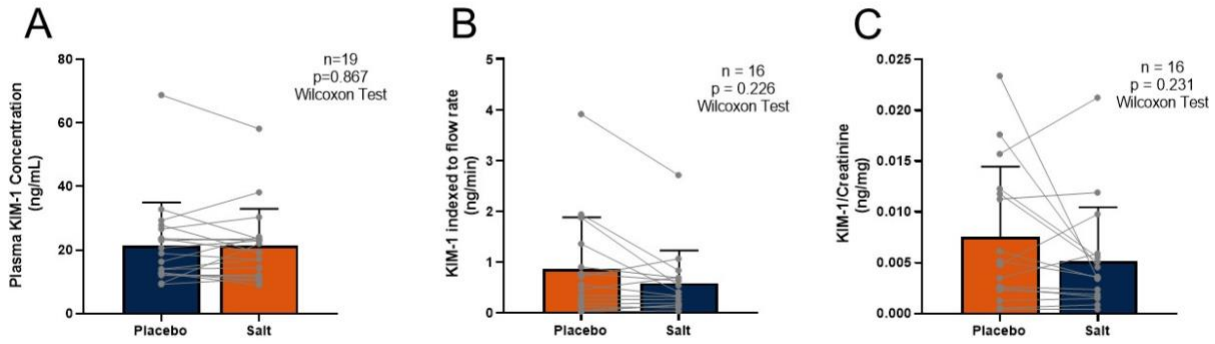

Figure 2. The effects of high dietary salt intake on KIM-1. (A) Plasma KIM-1 was not affected by salt loading. (B) Urine KIM-1 indexed to urine flow rate, and (C) KIM-1 indexed to urine creatinine concentration were not affected by salt loading. Sample sizes and statistical tests are reported in each graph. All data are presented as individual data points overlaid with mean ± SD.

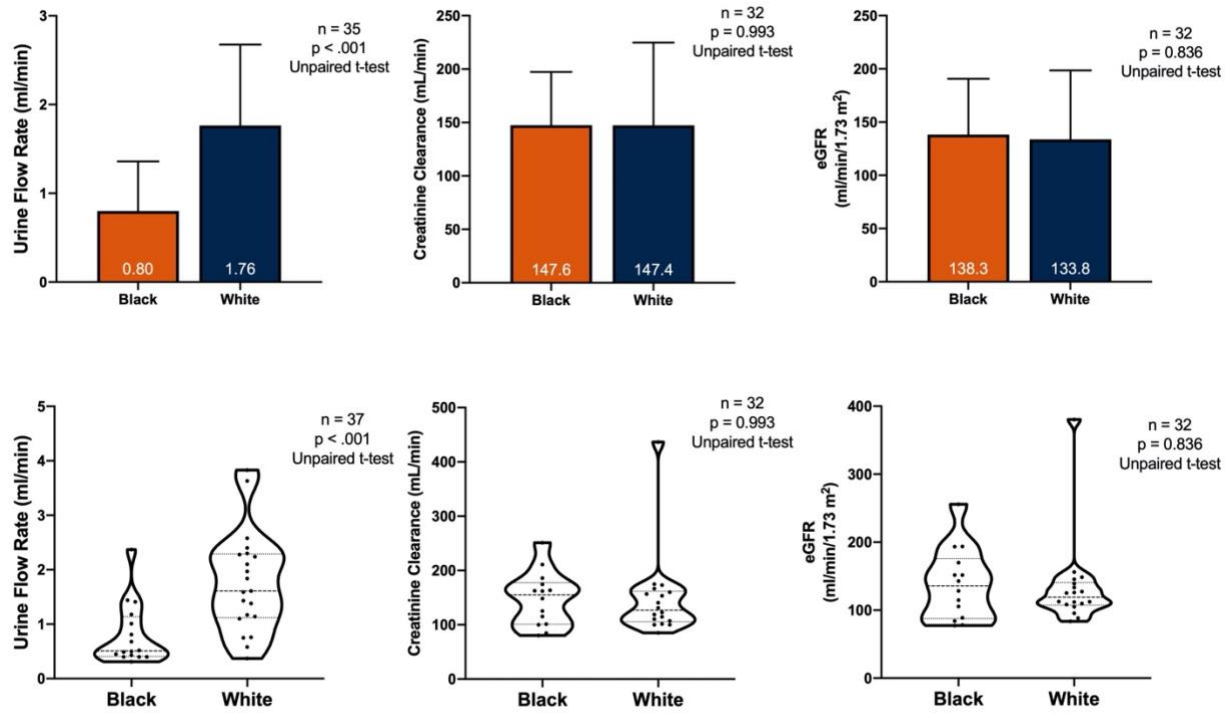

Figure 3. Racial differences in kidney function. (A) Urine flow rate was greater in the White cohort compared to Black cohort. (B) Creatinine clearance, and (C) eGFR were not different between groups. Sample sizes and statistical tests are reported in each graph. All data are presented as mean ± SD (top panel) or individual data points overlaid in a violin plot (bottom panel).

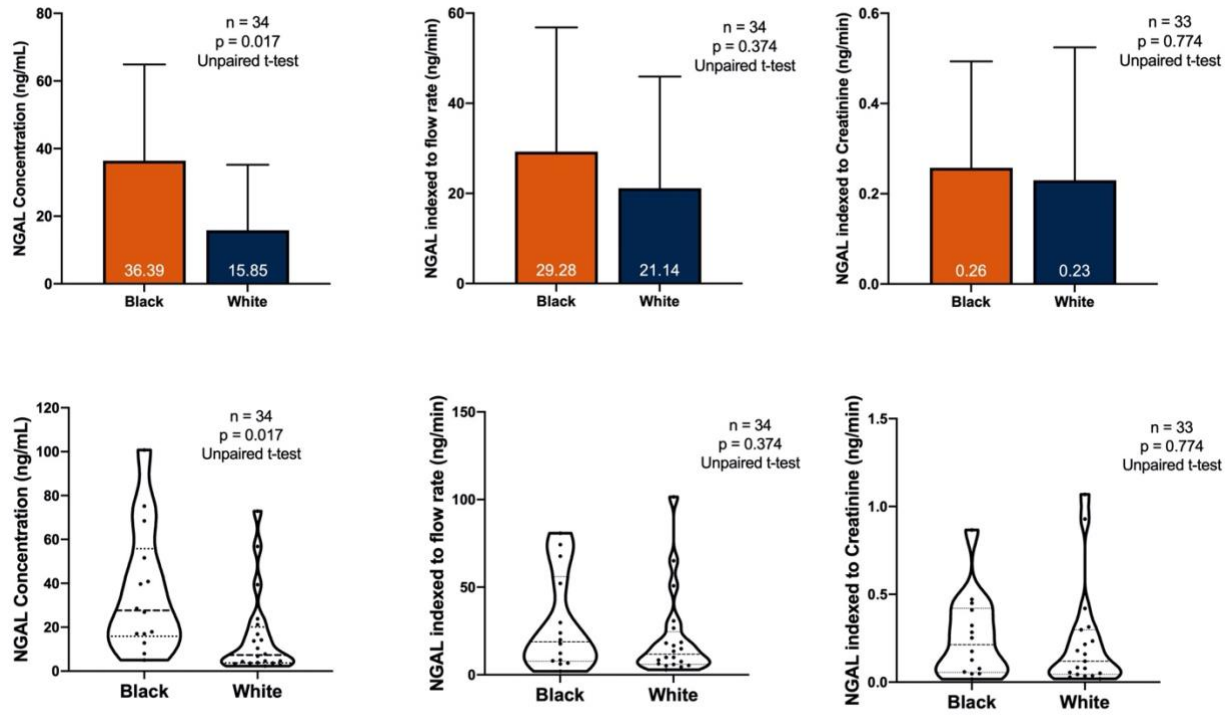

Figure 4. Racial differences on NGAL. (A) NGAL concentration was greater in the Black cohort compared to the White cohort. (B) Urine NGAL indexed to urine flow rate, and (C) NGAL indexed to urine creatinine concentration were not different between groups. Sample sizes and statistical tests are reported in each graph. All data are presented as mean ± SD (top panel) or individual data points overlaid in a violin plot (bottom panel).

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Appendix I Curriculum Vitae

Alex Barnett B.S.

Auburn University, School of Kinesiology,
301 Wire Rd. Auburn, AL 36849
904-554-9509
amb0127@auburn.edu

EDUCATION:

M.S. Auburn University – Auburn, AL
Kinesiology, Exercise Physiology
August 2019 – current
Thesis: *The Influence of Salt Loading on Kidney Injury Biomarkers in Healthy Young Adults*

B.S. Auburn University – Auburn, AL
Kinesiology, Exercise Science
August 2015 – May 2019

PROFESSIONAL EXPERIENCE:

Graduate Student Class Instructor
August 2019 – current
Auburn University - Auburn, AL
School of Kinesiology

Graduate Research/Teaching Assistant
August 2019 – current
Auburn University - Auburn, AL
School of Kinesiology
Neurovascular Physiology Laboratory

Graduate Research Assistant
May 2020 – April 2021
Auburn University - Auburn, AL
Neurovascular Physiology Laboratory
Funded by: NIH | National Center for Advancing Translational Sciences (NCATS): UL1TR003096
Project: *The Influence of Mitochondrial-Derived Reactive Oxygen Species on Racial Disparities in Neurovascular Function*

AWARDS & HONORS:

Graduate Teaching Assistantship
August 2019- current
Dean's List
Summer 2016

MANUSCRIPTS:

Accepted:

1. Linder BA, **Barnett AM**, Tharpe MA. Move it or lose it: Limb immobilisation results in impaired postprandial skeletal muscle glucose uptake. *The Journal of Physiology*. May 25. doi: 10.1113/JP281750. Online ahead of print.

In preparation:

2. **Barnett, AM**, Babcock MC, Watso JC, Migdal, KU, Gutiérrez OM, Farquhar WB, Robinson AT**.** The Influence of Salt Loading on the Kidney Injury in Healthy Young Adults. (In preparation, *Clinical Journal of the American Society of Nephrology*: May 2021)

CONFERENCE PRESENTATIONS:

1. **Barnett, AM,** Babcock MC, Watso JC, Migdal, KU, Farquhar WB, Robinson AT. The Influence of Salt Loading on Glomerular Hyperfiltration and Blood Pressure Variability in Healthy Young Adults. April 27 -30, 2021. EB 2021 Remote
2. Tharpe, MA, Hutchinson ZH, **Barnett, AM**, Linder BA, Robinson AT. Effects of MitoQ on Central Hemodynamics, Arterial Stiffness, and Oxidative Stress in Healthy, Young Adults. April 27-30, 2021. EB 2021 Remote
3. **Barnett, AM,** Babcock MC, Watso JC, Migdal, KU, Farquhar WB, Robinson AT. The Influence of Salt Loading on the Kidney Injury Biomarker Neutrophil Gelatinase-Associated Lipocalin in Healthy Young Adults. April 4 -7, 2020. San Diego, CA* Moved remote due to COVID-19
4. **Barnett, AM,** Babcock MC, Watso JC, Migdal, KU, Farquhar WB, Robinson AT. The Influence of Salt Loading on the Kidney Injury Biomarkers in Healthy Young Adults. March 31, 2020. Auburn, AL* Moved remote due to COVID-19

TEACHING EXPERIENCE:

Auburn University- Auburn, AL

- Class Instructor for PHED 1003, *Active Auburn* - Instructed an activities-based group fitness class. 08/2019 - current
- Class Instructor for PHED 1220, *Cardiorespiratory Fitness: Circuit Training* - Instructed an activities-based group fitness class. 08/2019 - 05/2020

CERTIFICATES & MEMBERSHIPS:

09/2019 – present Member - American College of Sports Medicine (ACSM)
09/2019 – present Member - American Heart Association (AHA)
09/2019 – present Member - The American Physiological Society (APS)
08/2019 – present American Heart Association certification in CPR & AED

COMMUNITY SERVICE:

06/2021	21st Century Summer Camps – Kinesiology (Auburn, AL)
06/2021	Duresville AME Clean Up Project (Macon, GA)
02/2021	Our House After School Outreach (Auburn, AL)
06/2020	East Alabama Food Pantry (Auburn, AL)
01/2018	Auburn Alternative Student Breaks – Martin Luther King Project (Selma, AL)
12/2016	Caring for Others Inc. Christmas Drive (Atlanta, GA)
12/2015	Caring for Others Inc. Christmas Drive (Atlanta, GA)

RELEVANT MEETINGS AND WORKSHOPS ATTENDED:

02/2020	Southeast American College of Sports Medicine. February 13-15, Jacksonville, FL
09/2019	University of Alabama at Birmingham (UAB) Center for Exercise Medicine 7th Annual Symposium. September 13, Birmingham, AL

INVITED ORAL PRESENTATIONS:

1. Overview of the Auburn University Neurovascular Physiology Laboratory. Spelman College, Environmental & Health Sciences Program (EHSP), EHSP day. Zoom Presentation April 22, 2021

Technical Skills:

- Laboratory: Enzyme-Linked Immunosorbent Assay (NGAL, KIM-1, IL-16, SOD, Albumin, Cystatin C, Urea, MCP-1), Data Analysis (Excel, GraphPad, LabChart), Prep and centrifugation of blood samples, Serum and Urine Analysis, Electron Paramagnetic Resonance sample prep, Peripheral Blood Mononuclear Cell isolation
- Clinical: FMD analysis of brachial artery, measure arterial stiffness via Sphygmocor XCEL, Phlebotomy trained, Photoplethysmography, Beat-to-Beat

blood pressure (Finapres), CPR certified, Determination of hematocrit and hemoglobin

- Administrative: Cold Call, Employee Hiring and Training, Fundraising, Communication Skills