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The Role Of Alström Syndrome 1 (alms1) In Hypertension, Salt Sensitivity And Metabolic Syndrome

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THE ROLE OF ALSTRÖM SYNDROME 1 (ALMS1) IN HYPERTENSION AND SALT SENSITIVITY AND METABOLIC SYNDROME

by

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DISSERTATION

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DEDICATION

To the loving memory of my Grandparents, your sacrifices have not been forgotten and the legacy of our families will continue to flourish.

To my parents, Mr. Robert L. King and Mrs. Nancy C. King, thank you for the love, thank you for the discipline, thank you for the motivation, and thank you for the support.

THANK YOU FOR EVERYTHING!

To my little sister, Karrisha N. King, thank you for being so supportive and for doing a wonderful job helping with our parents. Thank you for being such a great sister and being there to help with your niece and nephews. Thank you little sis, for being part of my success.

To my husband, Rafael Medina-Lopez, we have overcome so much together and as the Lord continues to guide our steps I know that our family will grow in strength, love and happiness.

Thank you so much for being a wonderful father to our children. Thank you for embarking on this journey with me…. Thank you for accepting ALL of me.

To my extended family and friends, thank you for the prayers, words of encouragement and support.

Most importantly, I must with the highest regard give thanks to the LORD, God Almighty! For none of this would be possible without the Lord's grace and mercy! Amen.

"O GIVE thanks unto the Lord; call upon His name; make known His deeds among the people." (KJV Psalm 105:1)

ALL GLORY BE GIVEN TO OUR HEAVENLY FATHER! Amen.

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PREFACE

The dissertation that follows is the result of my training as a graduate student in the laboratory of Dr. Pablo A. Ortiz at the Hypertension and Vascular Research Division, Henry Ford Hospital, Detroit MI. I came to Dr. Ortiz lab with prior experience conducting basic science research as an undergraduate student. However, I had no prior experience working with rodents. Before coming to the WSU School of Medicine Physiology Department I obtained a Master's of Science from the WSU Physician Assistant Program. My work experience as a Physician Assistant included surgery. When joining Dr. Ortiz's lab he was in need of a student to conduct whole-body experiments on the ALMS1 rats which included survival surgeries. So it was inevitable that my dissertation research became another fundamental component of this larger research project involving ALMS1. Data previously collected by former colleagues in the lab identified ALMS1 as one of the interacting partners with the carboxyl-terminus of NKCC2 (the carboxyl terminus is a domain important for NKCC2 endocytosis and is critical for the regulation of NaCl reabsorption by the kidney that ultimately influences the regulation of arterial pressure). In the years preceding the preparation of this dissertation, I contributed to another ongoing research project in the lab by performing several blood pressure telemetry studies which has resulted in a contributing author for the manuscript titled, "Fructose but not Glucose Induces Salt Sensitivity of Blood Pressure in Normal Rats" which is in the process of submission for publication. While contributing my time and skills towards the fructose project, I began to learn the background and significance of Alström Syndrome (ALMS). Though ALMS only affects a small percentage of the population, I found this rare autosomal recessive disease to be fascinating. More so, I found it peculiar that the prevalence is increasing which is likely a reflection of the medical community becoming

more knowledgeable about this medical condition. This sparked my interest utilizing the novel ALMS1 knockout (KO) rats created in our lab, to learn about the underlying cause(s) of hypertension, metabolic syndrome and renal disease which are common diagnosed in the ALMS human population. All data used in this thesis are either already published or are in the process of being published. This includes (but is not limited to) the characterization of endothelial, cardiovascular function in ALMS1 knockout rats, the results to the GST-pull down assays with carboxyl-terminus ALMS1 in rat thick ascending limb (TAL) lysates in which approximately 60 proteins were pulled down. Moreover, I contributed data and provided scientific input while providing rats from the ALMS1 rat colony (that I have managed for more than 1.5 years) for a former graduate student in the lab who has since defended: Dr. Ankita B. Jaykumar. Hence, I am a contributing author in her manuscript that was re-submitted for publication to JCI insight titled, “ALMS1 a Novel interacting protein of NKCC2 with a role on Renal Function and Blood Pressure”. I have also provided ALMS1 rats (KO and WT) for other colleagues in the lab (Dr. Mono Sumit and Mrs. D’Anna Potter to list a few) and hence will be a contributing author in the corresponding manuscripts. Furthermore, I have contributed data to another project led by my colleague, Dr. Cesar Romero, which has resulted in a co-author manuscript pending publication. Based on my independent research with the ALMS1 project, I will have two 1st author manuscripts that would have been submitted or in the process of submission to peer-reviewed journals (i.e. AJP renal) for publications. In summary, as a graduate student in Dr. Ortiz’s lab my contributions have supported the progression of the ALMS1 research. By working independently and as a team member, I will have several manuscripts either published or to be published (to peer-reviewed journals) as a graduate (PhD) student.

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LIST OF ABBREVIATIONS

ALMS1:	Alström syndrome 1 protein (Italicized version correspond to its gene or RNA)
ANOVA:	Analysis of Variance
βAR:	β adrenergic receptor
BL:	Baseline
BP:	Blood pressure
BW:	Body weight
C2-NKCC2:	A region in the carboxyl-terminus of NKCC2
CA:	Carbonic anhydrase
CR:	Caloric restricted
CKD:	Chronic kidney disease
DBP:	Diastolic blood pressure
DOB:	Date of birth
ENaC:	Epithelial Na channel
GFR:	Glomerular filtration rate
GLUT4:	Glucose transporter-4
GST:	Glutathione S-transferase
GTT:	Glucose tolerance test
GWAS:	Genome-wide association studies
HCTZ:	Hydrochlorothiazide diuretic
HDL:	High density lipoproteins
Het:	Heterozygous
IACUC:	Institutional Animal Care and Use Committee
IP:	Intraperitoneal
KW:	Kidney weight

KO:	ALMS1 Knockout
LC:	Liquid chromatography
MAP:	Mean arterial pressure
MBP:	Mean blood pressure
MS:	Mass spectrometry
Na:	Sodium
Na^{+}/K^{+}/ATPase:	Sodium-potassium pump
NHE:	Na/H exchanger
NHGRI:	National human genome research institute
NIH:	National Institutes of Health
NKCC2:	$Na^{+}/K^{+}/2Cl^{-}$ co-transporter
NRK:	Normal rat kidney
PKA:	Protein kinase A
RBF:	Renal blood flow
RGD:	Rat genome database
RBP:	Renal blood flow
ROMK:	Renal outer medullary K^{+} channel
RVR:	Renal vascular resistance
SBP:	Systolic blood pressure
SD:	Sprague-Dawley
SNP:	Single nucleotide polymorphism
SSBP:	Salt sensitive blood pressure
TAL:	Thick ascending limb
TC:	Tail-cuff
TGF:	Tubulo-glomerular feedback

TGN:	Trans-golgi network
WT:	Wild-type Dahl SS

CHAPTER 1: BACKGROUND, GENERAL HYPOTHESIS AND SPECIFIC AIMS

Human anatomy of the kidney

The kidneys are vital organs that function to filter the blood by removing wastes and fluid while maintaining levels of important electrolytes in the human body circulation. Each kidney contains about 1 million structures called nephrons. Each nephron starts with a filter called the glomerulus which is surrounded by the Bowman's capsule which facilitates the filtration of blood to form urine. The filtrate then moves along the nephron via segments of the tubule starting with the proximal convoluted tubule → proximal straight tubule → Loop of Henle (thin descending, thin ascending and thick ascending) → distal convoluted tubule → connecting tubule → collecting duct → forming urine that goes to the ureters (Figure 1) [18, 69, 75, 95]. These nephron segments have different absorptive and secretory properties that are a result of several mechanisms including transporters that function to move ions across the apical and basolateral membranes [18, 69, 75, 95]. The kidney is a key component for maintaining homeostasis of the blood pH, osmolality, as well as ion and water composition in the body. The ability of the kidneys to filter blood is important for removing metabolites, ions and osmolytes from circulation. Moreover, the kidney secretes several vasoactive substances such as the renin-aldosterone-angiotensin-system (RAAS), which has been known to influence the excretion and reabsorption of electrolytes, as well as fluid along the nephron. This will affect the fluid volume in the extracellular compartments which is a determinant of blood pressure.

When discussing blood pressure there is one nephron segment that is of much importance and that is the Loop of Henle, which extends deep into the renal medulla. The most distal portion of the Loop of Henle is called the thick ascending limb (TAL), which is divided into medullary and cortical regions. The TAL can reabsorb approximately 25-30%

of NaCl via the Na-K-2Cl cotransporter termed NKCC2 which has been implicated in the development of hypertension [6-8, 18, 69, 75].

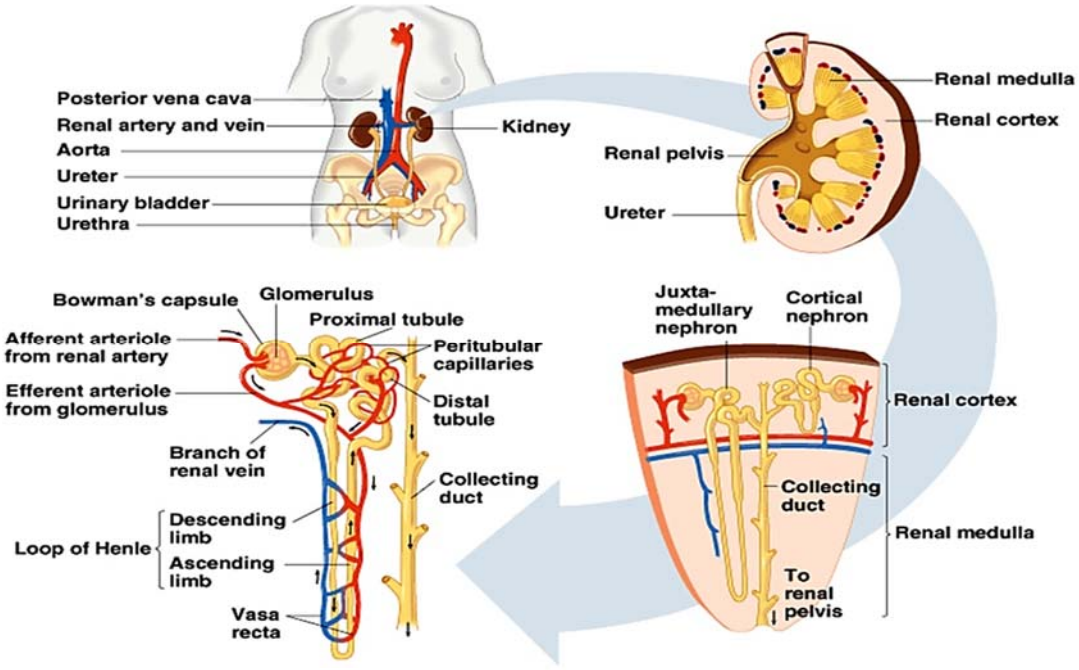


Figure 1. Diagram of a Human Nephron Kidney and Nephron | Urinary System, Pearson Education Inc. Jan 17, 2018.

Role of NKCC2 in NaCl reabsorption by the thick ascending limb (TAL)

The TAL contributes to the maintenance of the extracellular fluid by reabsorbing 25- 30% of filtered NaCl load from the glomerulus which decreases the luminal Na+ concentration from approximately 140 mM in the inner stripe of the outer medulla to 30–60 mM at the macula densa [7, 95]. TAL also plays an important role in the concentration of urine by actively reabsorbing NaCl via the NKCC2 cotransporter and being water-impermeable. These characteristics of the TAL dilutes the luminal fluid and drives the counter current multiplication which generates the osmolality gradient in the outer medulla that is required in order for vasopressin to facilitate absorption of water by the collecting duct (water permeable) [6-8, 18, 69, 75]. Also, the TAL reabsorbs other ions as well, such as K^+, Mg^{2+} and Ca^{2+} ions.

The TAL functions to establish a hyperosmotic interstitium which at the collecting duct drives the reabsorption of water through osmotic forces. The TAL and the rest of the nephron, have epithelial cells that line the membrane and they have an apical-basolateral polarity.

As previously described by my colleague [6-8, 69], on the apical surface, $Na^+/K^+/2Cl^-$ co-transporter (NKCC2) and Na-H exchanger (NHE3, not shown in Figure 2) reabsorb Na^+, K^+ and Cl^- ions from the lumen by utilizing the electrochemical gradient generated by the sodium-potassium pump (Na^+-K^+ ATPase) in the basolateral membrane. Na+ exits the cell at the basolateral side through the Na^+-K^+ ATPase while the Cl^- exits through the Cl^- channels and K^+/Cl^- co- transporter [6-8, 23, 69].

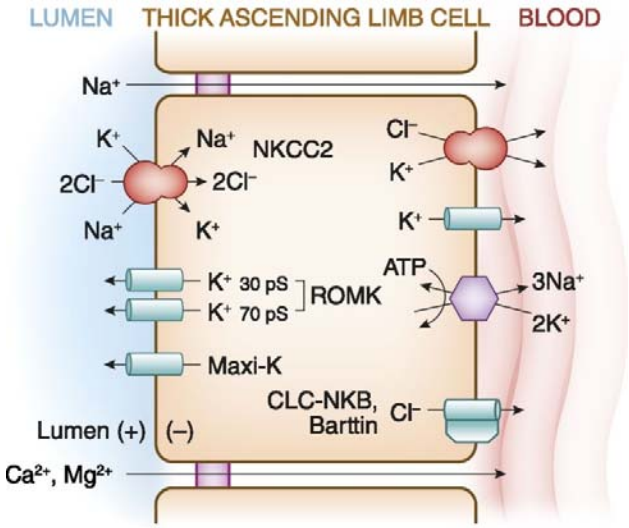


Figure 2. Representation of NaCl reabsorption by the thick ascending limb. (Clinical Journal of the American Society of Nephrology)

The K^+ ions are then recycled back into the lumen via the renal outer medullary potassium channel (ROMK) which creates a positive luminal electric potential which allows the paracellular pathway to drive Na^+, Ca^{2+} and Mg^{2+} absorption [7, 23, 69, 128]. These mechanisms reinforce the notion that TAL plays a crucial role in the regulation of water and ion reabsorption in the kidney. The TAL will therefore influence urine concentration and arterial pressure regulation.

Control of blood pressure: role of NKCC2

Bartter syndrome type I is characterized by polyuria, inability to concentrate urine and hypotension and is caused by loss of function secondary to mutations in NKCC2 [2, 12, 16-17, 69, 87, 115]. Mice models with genetic deletion of NKCC2 recapitulate Bartter syndrome type I phenotype is associated with severe volume depletion and low BP [14]. Two independent groups have shown that mutations in NKCC2 decrease NKCC2-mediated NaCl reabsorption and blood pressure in humans [2, 7, 16, 22]. Also, an enhanced NKCC2-mediated NaCl reabsorption is associated with salt-sensitive hypertension in animal models and in humans [3, 24, 78]. There are loop diuretics, such as furosemide and bumetanide that selectively inhibit NKCC2 activity and are used clinically for the treatment of hypertension. Thus, NKCC2-mediated NaCl absorption by the TAL is crucial in regulation of blood pressure [45, 77].

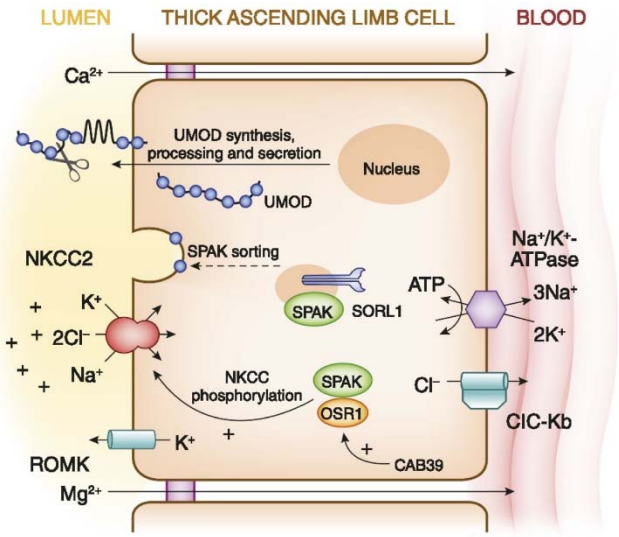


Figure 3. NKCC2 phosphorylation by SPAK/OSR1 in the thick ascending limb. (Clinical Journal of the American Society of Nephrology)

NKCC2: insight on protein-protein interactions that play a role in regulating NKCC2 endocytosis

NKCC2-mediated NaCl reabsorption is regulated by several factors: 1) phosphorylation at threonine (96/101) by STE20- and SPS1-related proline and alanine-rich kinases (SPAK) and oxidative stress-responsive kinase 1 (OSR1) and phosphorylation at Ser (126) site by PKA, 2) protein-protein interactions and 3) protein

trafficking determining NKCC2 apical abundance [43, 46, 69, 77, 100, 101, 103, 105, 125]. Though NKCC2 phosphorylation has been well studied; the mechanisms and protein-protein interactions regulating NKCC2 apical trafficking a still to be determined.

While most NKCC2 is located sub-apically as visualized by electron microscopy, our lab previously showed that a steady-state, only a small fraction (~5%) of the total NKCC2 is targeted to the apical surface of the TAL cells [7-8, 23]. Steady-state surface NKCC2 levels are maintained by a balance between various protein trafficking modalities such as exocytic delivery, endocytosis and recycling together determining NKCC2 activity (Figure 4) [7-8, 99, 101]. Our lab previously showed that blocking NKCC2 endocytosis leads to increased NKCC2 levels at the apical surface and increased NKCC2-mediated NaCl absorption in the TAL [101]. Additionally, surface NKCC2 was found to be significantly higher in a rat model of salt-sensitive hypertension [77]. These observations demonstrate an important relationship between regulation of NKCC2 abundance at the apical membrane by NKCC2 endocytosis and NKCC2-mediated NaCl reabsorption by the TAL. This also highlights the importance of NKCC2 endocytosis as a mechanism to regulate renal function and blood pressure.

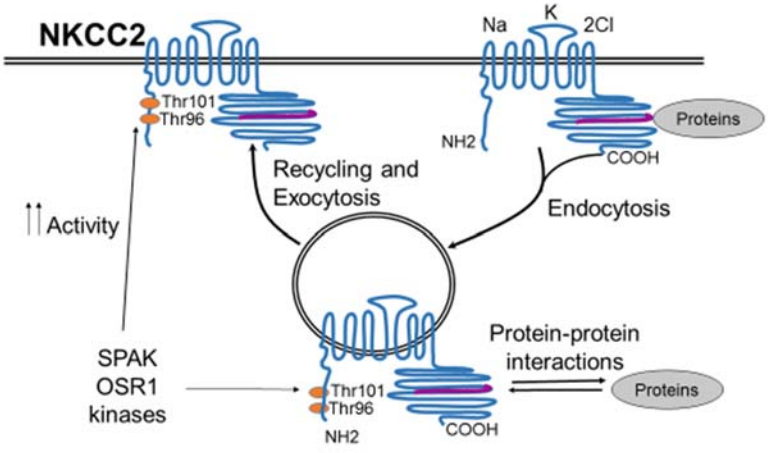


Figure 4. Trafficking, protein-protein interactions and phosphorylation control NKCC2 activity at the membrane.

The molecular mechanisms that regulate NKCC2 endocytosis remain unclear. Protein-protein interactions with NKCC2, such as myelin and lymphocyte-associated protein (MAL/VIP17), have been described to regulate NKCC2 endocytosis [7, 26, 49, 69]. Previously, only four proteins were known to bind the ~ 400 amino acid long, intracellular carboxyl-terminus of NKCC2 (C2-NKCC2) which consists of a unique 71 amino acid stretch shown to be important for NKCC2 apical trafficking [7, 26, 69]. My former colleagues, Drs. Jaykumar and Caceres, used a proteomics approach via a glutathione-S-transferase (GST) fusion protein with C2-NKCC2 as bait, to pull down proteins from thick ascending limb lysates. Mass spectrometry was used to identify new C2-NKCC2 interacting partners that could play a role in NKCC2 endocytosis, regulation of blood pressure and renal function (part of this work was published in Ankita B. Jaykumar dissertation and is currently being considered for publication) [69]. Alström syndrome 1 protein (ALMS1) was one of sixty (60) proteins pulled down with the C2-NKCC2, and later confirmed to be an interacting partner [69].

ALMS1 gene

Alström syndrome an autosomal recessive disease caused by mutation that have been mapped to chromosome 2p13 [31]. Hearn et al., identified this disease as being caused by mutations in a large gene on chromosome 2 in which several patients with similar phenotypic characteristics [52]. This is a large gene of 12.9-kilobase that encodes for a protein of 4,169 amino acids. The protein contains a large tandem-repeat domain (also known as ALMS repeats) made of 34 imperfect repetitions of 47 amino acids [52]. To date several mutations have been identified involving this gene that can lead to this disease. And it's important to note that the expression of the ALMS1 gene can be found in several tissues such as the heart, brain, pancreas, liver, kidney and reproductive organ

systems [9, 33, 48, 54, 84-85, 87].

Alström syndrome (ALMS): in the human population

In humans, mutations in the ALMS1 gene causes Alström Syndrome (ALMS), which is a rare inherited autosomal recessive disease characterized by hypertension (HTN), obesity, insulin resistance and varying degrees of neurological abnormalities [84, 86, 88]. Recently, some genome-wide-association studies (GWAS) have linked eighty (80) ALMS1 single nucleotide polymorphisms (SNPs) in the general population with decreased renal function and renal disease [39, 80, 102]. Patients with this progressive multi-systemic disorder are at risk of developing multiple co-morbidities such as cardiomyopathy, insulin resistance, and end-stage renal disease (ESRD) [88-89]. There are approximately 50% of ALMS patients who develop progressive renal impairment involving varying degrees of glomerular disease (along with impaired glomerular filtration rate) and albuminuria which may lead to the onset of HTN [88].

The ALMS1 gene has been associated with HTN in some patient populations and is associated with lower renal function according to GWAS [103]. Others have also shown that ALMS patients have mutations in the ALMS1 gene and are hypertensive; they also exhibit a progressive decline in renal function and/or renal disease [84-85]. Yet, the role of the ALMS1 in hypertension, salt sensitivity and renal salt absorption has not been determined. And now that clinicians are more knowledgeable about this medical condition the number of reported cases is rising and foundations like Alström Syndrome International (ASI) serve as a reminder of the higher number of individuals affected by this disease [57].

ALMS1 gene in animal studies

There are other scientists who have taken an interest in the study of ALMS1 and how it's involved in metabolic abnormalities. There are currently several other laboratories

that utilize a mouse model with genetic mutation and alteration of the ALMS1 gene. These mice have developed characteristics similar to those seen in patients with ALMS. Collin et al. generated an ALMS1 KO mice model and their research revealed that these mice (like the ALMS patients) developed obesity, hypogonadism, hyperinsulinemia, retinal dysfunction and late-onset hearing loss [33]. Then Arsov and colleagues conducted experiments to describe the phenotypic characterization of a new mouse model of Alström syndrome called "fat aussie" [9]. And in comparison to the ALMS1 mutant mice model mentioned earlier, these fat aussie mice also have ALMS1 mutations and they develop obesity, glucose intolerance and hypercholesterolemia, etc. [9]. However, no research has been conducted in a rat model with the genetic deletion of ALMS1.

Role of ALMS1 in hypertension, renal and metabolic function

Deletions and mutations in the ALMS1 gene is associated with Alström syndrome. As previously mentioned, this is medical disease characterized by hypertension, obesity, insulin resistance, kidney dysfunction and chronic kidney disease [84, 86, 88]. Moreover, clinicians such as Marshall et al., were one of the first to characterize ALMS patients and it was found that these patients had a reduced glomerular filtration rate (GFR) and albuminuria indicative of renal impairment. There is a correlation between aging and renal disease. And the most common cause of death in ALMS patients is end-stage renal disease [86-88].

Two independent genome-wide association studies discovered single nucleotide polymorphisms (SNPs) in ALMS1 locus associated with GFR and chronic kidney disease [102, 105]. Furthermore, human chromosome 2 has been consistently identified as a genomic region with genetic linkage suggesting that one or more loci contribute to blood pressure and hypertension [13]. Barkley et al. demonstrated that the ALMS1 gene has

been linked to hypertension status in a “multipoint linkage analysis” in primary sibling samples of African American, Caucasian and Mexican population [13]. In this population analysis, seven single nucleotide polymorphisms in ALMS1 were associated with hypertension and increased pulse pressure [88].

Nonetheless, ALMS1 has been linked to both hypertension and metabolic syndrome in humans. Patients with mutations in ALMS1 are obese with hyperlipidemia and insulin resistance. ALMS1 mutant mice are obese and exhibit most features of metabolic syndrome, but their blood pressure has not been measured. Marshall, et al. described that obesity, hyperinsulinemia and hypertriglyceridemia are clinical manifestations that occur in the human ALMS population [88-90]. The fat aussie mice and ALMS1 KO mouse model supports the description of the phenotypic characteristics noted for the ALMS1 patients [9, 33]. However, until now research on the renal mechanisms that mediate hypertension involving ALMS1 has not been done. And the mechanism by which ALMS1 affects renal Na+ handling, blood pressure, as well as salt-sensitivity is unknown.

Defining the WT (Dahl) salt sensitive rat control group

For this research we utilized wild-type (WT) dahl-salt sensitive rats for the control group. This is important because it is known that dahl-salt sensitive rats are not ordinary controls. These rats have an impaired pressure-naturiesis relationship (resembling that of salt-sensitive individuals in the human population) which occurs in response to the kidneys inability to excrete sodium [15, 49]. Therefore, the dahl-salt sensitive rats become hypertensive when fed a high salt [6, 49, 50, 110]. More so, the dahl-salt sensitive rats have been shown to have enhanced NaCl reabsorption along the nephron segment called the thick ascending limb (TAL) which is related to an elevation in blood pressure [6, 50, 110]. The NaCl reabsorption along the TAL is mediated via the Na+-K+-2Cl- cotransporter termed,

NKCC2, located along the apical membrane surface of the TAL. However, not only are the dahl-salt sensitive rats hypertensive, but also they have been shown to develop impaired renal function and metabolic syndrome over time [42, 49, 50, 110]. It is for these reasons we chose to use dahl-salt sensitive rats as our control group. The ALMS1 KO rats used for our research have been generated in a (dahl) salt sensitive background; and we believe that the deletion of ALMS1 causes an acceleration of hypertension, as well as enhanced salt sensitivity and an earlier onset of metabolic alterations.

General hypothesis and specific aims

Specific Aim 1: The genetic deletion of the ALMS1 gene in rats causes a salt-sensitive increase in blood pressure by increasing renal NaCl reabsorption by stimulation of NKCC2 in the thick ascending limb.

Hypothesis 1: The product of the ALMS1 gene is involved in thick ascending limb (TAL) NaCl reabsorption via inhibition of NKCC2. Thus, we hypothesize that the deletion of ALMS1 decreases the ability of the kidney to excrete Na and increases blood pressure or enhances salt sensitivity.

Rationale: Enhanced NaCl absorption by the TAL is associated with hypertension in humans and animals. The co-transporter NKCC2 is the predominant pathway for NaCl absorption in the TAL. Previous findings from this lab, suggest that ALMS1 is expressed in TALs and binds NKCC2 implying that this gene may be involved in NaCl reabsorption by the kidney. In order to determine if ALMS1 is involved in blood pressure regulation we will obtain blood pressure measurements at various dietary Na intakes using both invasive radio-telemetry and noninvasive tail cuff blood pressure measurements in younger 7-9 week old male rats. To further explore the renal Na handling in male ALMS1 KO rats we will perform metabolic cage experiments using several diuretics targeting different

segments of the nephron (such as the proximal tubule, thick ascending limb of loop of Henle, distal convoluted tubule and collect duct).

Specific Aim 2: The hypertension and NKCC2 activation in ALMS1 Knockout (KO) rats is independent of obesity.

Hypothesis 2: The hypertension observed in the ALMS1 KO rat is in part due to enhanced NaCl reabsorption by the TAL and is independent from an increase of body weight for this rat model.

Rationale: In order to determine if ALMS1 deletion is the contributing factor for the onset of hypertension and salt-sensitivity in male ALMS1 KO rats we will exclude obesity as another potential factor for the elevation of blood pressure in this rat model. We will subject ALMS1 KO rats to caloric restriction to decrease the body weight (without constricting growth) and then measure their blood pressure and NKCC2 activity. Therefore, our research will allow us to determine how the ALMS1 protein with or without the presence of metabolic syndrome can influence NKCC2 function, especially in regards to inducing salt-sensitive hypertension.

CHAPTER 2: HYPERTENSION AND SALT SENSITIVITY IN THE ALMS1 KNOCKOUT RAT

Introduction

Hypertension, also known as high blood pressure, is defined as a chronic elevation of arterial blood pressure. Approximately 1 out of 3 U.S. adults has hypertension which is equivalent to 75 million people in this country [57]. Hypertension is the leading cause of cardiovascular disease and stroke which are both considered leading causes of the death [79]. Hypertension has been referred to as a silent killer because there are no identifiable symptoms and most individuals do not know they have this medical condition [57]. Per the CDC, hypertension data site, upon being diagnosed with hypertension only half of these patients have controlled blood pressure, <130/80 mmHg) [57]. Yet, the underlying etiology of hypertension remains unknown.

There have been many researchers determined to uncover the underlying mechanism for the development of hypertension. Based on prior literature, dietary sodium intake has been linked to the onset of hypertension [24]. The prevalence of hypertension is increasing and yields a higher rate of diagnosis within certain populations such as Black (African) Americans and Hispanic (Latino) Americans [36]. For instance, Dr. Weinberger et al. found that the general hallmark of hypertension (i.e. a change in blood pressure in response to changes in salt and water balance) is present in 73% of blacks with hypertension and 36% of blacks who are normotensive in which these individuals may also have a genetic contribution to salt sensitivity [126]. The American Heart Association defined salt sensitivity of blood pressure (BP) as a physiological trait present in rodents and other mammals, including humans, by which the BP of some members of the population exhibits changes that parallel changes in salt intake [36]. Animal models have

been used for many years to study hypertension and salt sensitivity in aims of improving the medical outcome for hypertensive patients, as well as those diagnosed with resistant hypertension [34].

In order to study the role of ALMS1 in salt sensitive hypertension and renal NaCl absorption, in collaboration with the rat genome editing consortium at the Medical College of Wisconsin (MCW), we generated the ALMS1 knockout (KO) rats in a Dahl salt-sensitive genetic background via a zinc finger nuclease genome targeting. PCR experiments were done to identify three strains of rats and then label them as ALMS1 KO vs. WT (Dahl) SS vs. Heterozygous (Het). The deletion of ALMS1 in the rat thick ascending limb (TAL) of the ALMS1 KO rats was confirmed using western blotting. Others have shown that deletions in the ALMS1 gene have been associated with characteristics similar to those seen in patients with ALMS. Collin et al. generated an ALMS1 KO mouse model and their research revealed that these mice (like the ALMS patients) developed obesity, hypogonadism, hyperinsulinemia, retinal dysfunction and late-onset hearing loss [33]. And as previously stated, the fat aussie mice were found to be obese, with glucose intolerance and hypercholesterolemia, etc. [9]. So our laboratory became interested in the study of ALMS1, and hence decided to utilize our novel ALMS1 Knockout (KO) rat model to determine if there is an effect on blood pressure and renal sodium handling.

Methods

Blood pressure measurements

Non-invasive tail-cuff plethysmography

The first technique used to measure blood pressure is non-invasive tail-cuff plethysmography. Our laboratory utilizes the Kent scientific (Torrington, CT 06790, USA) tail-cuff plethysmography to obtain blood pressure (BP) measurements for the rats. The

rats are placed inside the Kent Scientific rat holders which vary in size based on the rats body weight (BW). All rats are trained over a period of 1-2 weeks. The rats are grouped as ALMS1 KO (KO) versus WT (Dahl) SS (WT). When collecting the SBP measurements the KO rats are placed on one warmer panel while the WT rats are placed on another warmer panel. There are usually 1-2 cycles of measurements in which there are approximately 13 runs per cycle. The rat tail temperatures are recorded before each run and were usually in the range of 32-34 °C. Blood pressure measurements frequency varied based on the protocol, but were usually done every Monday-Wednesday-Friday.

Invasive radio-telemetry

The second technique used to measure blood pressure in conscious rats is invasive radio-telemetry. Our laboratory uses the Data Science International (DSI, St. Paul, MN 55112) HD-S10 telemetry catheters to collect information on several parameters such as, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR).

This surgery is performed using aseptic techniques to implant the radio-telemetry device in the right femoral artery of the rats after they've been anesthetized with a mixture of xylazine and ketamine given via an intra-peritoneal (i.p.) injection. DSI recommends using rats that weigh at least 175 g. Our rats weighed in the range of 175-200 g at the time of surgery. After the rats are anesthetized, they are placed on a heated surgical plate (in which the heat pad is pre-warmed and inserted underneath the surgical plate). The rats are prepped via hair removal at surgical site that is then dusted to ensure hairless surface and cleaned with betamide. Proper drapery is then placed. A surgical incision in the left inguinal area is made with a scalpel and the adipose tissue and fascia is divided to obtain a visual of the nerve, artery and vein (aka NAV) anatomy of the rat. The femoral artery is carefully

isolated before a small cut (using a 25-G needle) is made in the femoral artery to insert the catheter. The catheter is then inserted and advanced into the abdominal aorta. A hand-held radio on the AM station can detect the transmitter pulsatile frequency and confirm that the catheter is properly placed. Lastly, using blunt scissors, the transmitter is placed subcutaneously in the left flank area and secured with vet bond glue.

Our rats were allowed 5-7 days for recovery before recording the measurements to be used for the radio-telemetry data. Data was collected through DSI software, DATAquest ART, then transferred to PONEMAH (DSI software) for analysis before exporting to Microsoft excel. The measurements reported are average readings from SBP, MBP, DBP and HR for the two groups (KO and WT) over the duration of the experiment. Data was obtained for each individual rat before grouping.

Metabolic cage urine collection

Urine and ion excretion measurements: We used 7-9 week old male rats to determine which nephron segments are involved in the hypertension and salt sensitivity observed in the ALMS1 KO rats. The rats were placed in metabolic cages with free access to food and water. Also, because we found our ALMS1 KO rats to be hyperphagic, we pair-fed these rats by giving 20 grams of regular chow (containing 0.4% Na) daily. The rats were allowed to adjust to the new cage for 3-4 days before the urine samples were collected. Urine volume was recorded and urine osmolality was measured by freezing point depression with Advanced Model 3300 Micro Osmometer (Advanced Instruments Inc. Norwood, MA) [67]. Urine Na+ was measured in Nova 1+ Analyzer (Nova Biomedical, Waltham, MA) [67]. For diuretic response experiments, the rats were trained for 5 days to eat 1 gram of non-fructose containing chocolate frosting within 30 minutes after 8 hours of fasting. For the 1% NaCl load of body weight protocol, in order to ensure the rats ate their

3 grams of non-fructose containing chocolate frosting within 30-45 minutes, the rats were fasted for 16 hours. On day 5, the baseline urine was collected for 24 hours to record urine volume, urine osmolality and urine Na+ excretion. On the day of the experiments for diuretic response, the diuretics were weighed and mixed with the chocolate frosting and urine was collected to measure urine volume, urinary sodium content. Urine osmolality over a period of 12 hours after the rats were treated with the given diuretic. The diuretics were given 2-3 days apart to avoid cross-interference or discrepancies in the data.

Body weight measurements

The rats were placed inside a stainless steel container (and the scale was set to 0 grams) to obtain body weight in grams (g). Data was recorded in excel spread sheets and then transferred to GraphPad PRISM for graphing and data analysis.

Data analysis, justification of sample size and animal number

Statistical Analysis: For all statistical methods described below, we determined whether the data satisfy the assumptions for the procedure.

PRISM GraphPad software was used for statistical analysis of the data. One-way ANOVA or unpaired t-test was used to compare two (2) groups on an individual time point to examine the difference between the two means. 2-Way ANOVA was used when comparing mean values for more than two groups. Paired t-test was used to compare the differences in mean within the same group before and after treatment.

Justification of Sample Size: For all sample size calculations, we used an effect size of 1.7-1.8.The effect size was calculated from the difference between the means divided by the standard deviation. The magnitude was chosen based on previous work by our group with these models. Power was set at 80% and a two-sided test was always considered. ANOVA with repeated measures: Paired-testing was conducted using an

appropriately adjusted alpha level.

Results

ALMS1 knockout (KO) rats are hypertensive (HTN) and salt-sensitive with invasive radio-telemetry

In order to determine if the deletion of ALMS1 would influence blood pressure regulation in rats; invasive radio-telemetry was used to obtain blood pressure measurements in the unrestrained rat. For this protocol we used seven (7) younger male rats with an average age of 9 weeks old for each group (KO vs. WT) to measure blood pressure (BP).

Blood pressure (BP) measurements were continuously recorded over a period of approximately two months. The rats received a 0.22% sodium (Na) chow and regular water daily for more than a week at which time blood pressure (BP) measurements were obtained during the awake and rest cycle. Rats are a nocturnal species and the awake phase was during the hours of 8pm-8am which were further supported by the activity recordings for the 24-hours of telemetry BP measurements. In awake rats, the ALMS1 KO group maintained a higher systolic blood pressure (SBP) than the WT group while receiving both the normal (0.22 Na) and high salt (4% Na) chows (Figures 5-6). Thereby suggesting that

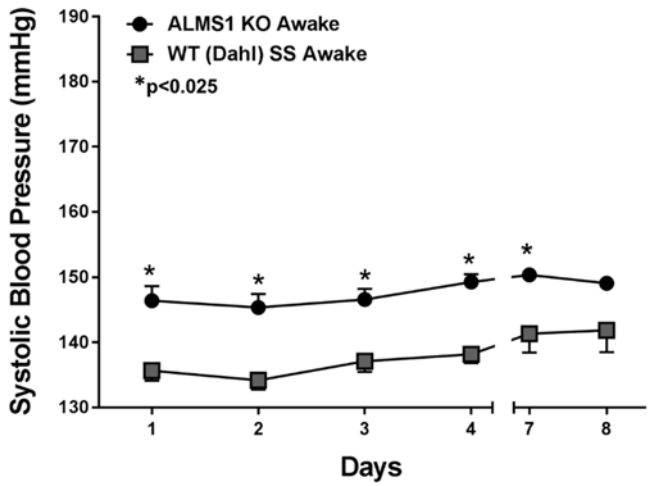


Figure 5. Systolic blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 0.22% Na (normal salt) chow using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the normal salt diet (KO: 147.9 ± 0.8 vs. WT: 138.1 ± 1.3 mmHg, p<0.025).

the ALMS1 KO rats are hypertensive and while receiving the same dietary Na chow, the ALMS1 KO rats have a higher SBP compared to the WT (Dahl) SS rats.

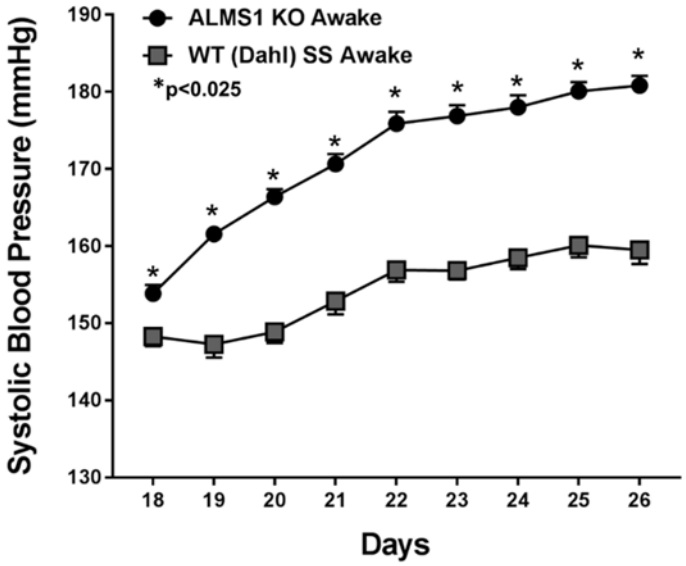


Figure 6. Systolic blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the high salt diet (KO: 171.6 ± 3.1 vs. WT: 154.3 ± 1.7 mmHg, $p<0.025$).

More so, both groups had an increase in BP measurements for in response to increasing the dietary Na intake from .22% to .4% (Figure 7. KO: 35 ± 2 vs. WT: 25 ±1 mmHg, $p<0.025$). Then when giving the loop diuretic, bumetanide (NKCC2 inhibitor) to the ALMS1 KO and WT (Dahl) SS rats dosed at 3mg/kg daily over 6 days our results showed that the ALMS1 KO rats maintained a higher SBP (Figure 8) and there was an equivalent decrease in BP for both groups (Figure 9, KO: 16±3 and WT: 15±2 mmHg, p=N.S.). The data in Figure 6 demonstrates that both groups are salt sensitive; but the ALMS1 KO rats have a greater response to salt intake compared to the WT (Dahl) SS rats.

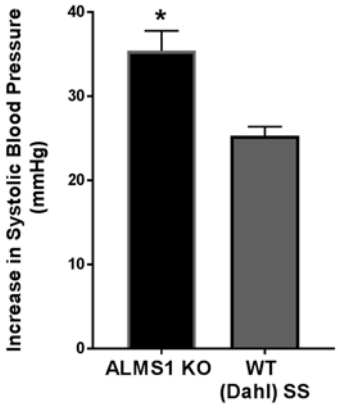


Figure 7. Differences in systolic blood pressure (SBP) in response to increasing the dietary sodium (Na) intake. ALMS1 KO rats systolic BP (SBP) have a significant increase in SBP compared to the WT (Dahl) when increasing dietary Na from 0.22 % to 4 %, (KO: 35 ± 2 vs. WT: 25 ± 1 mmHg, *p< 0.025).

Whereas, Figure 7 show that bumetanide resulted in an equivalent decrease in SBP for both groups in which KO rats did not have restoration of their SBP back to baseline SBP values. This suggest that both groups have increased NKCC2 activity; however, NKCC2 alone is not responsible for the elevation of blood pressure in response to increased dietary Na intake in ALMS1 KO rats.

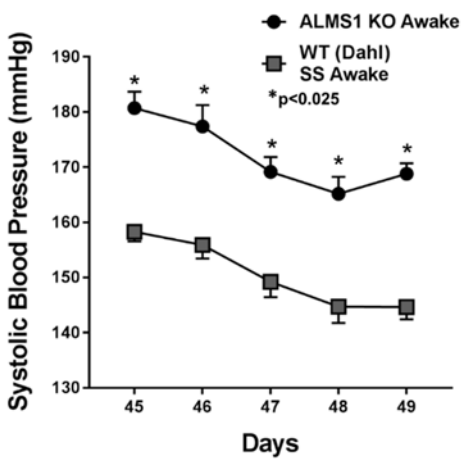
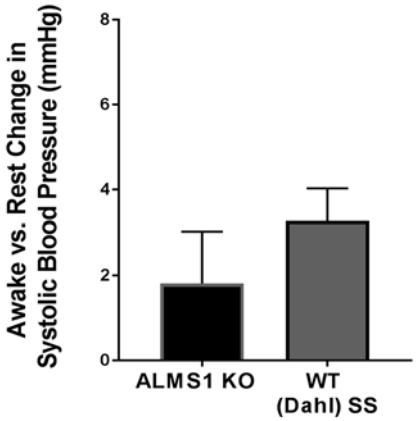


Figure 8. Systolic blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow and bumetanide (loop diuretic) using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT rats with the high salt diet and bumetanide (KO: 172.3 ± 2.9 vs. 150.6 ± 2.8 mmHg, p<0.025).

Figure 9. Differences in systolic blood pressure when receiving a 4% NA chow and then given bumetanide 3mg/kg daily. There was no difference in the decrease in SBP between both groups in response to bumetanide (KO: 16 ± 3 vs. WT: 15 ± 2 mmHg, p=N.S.)



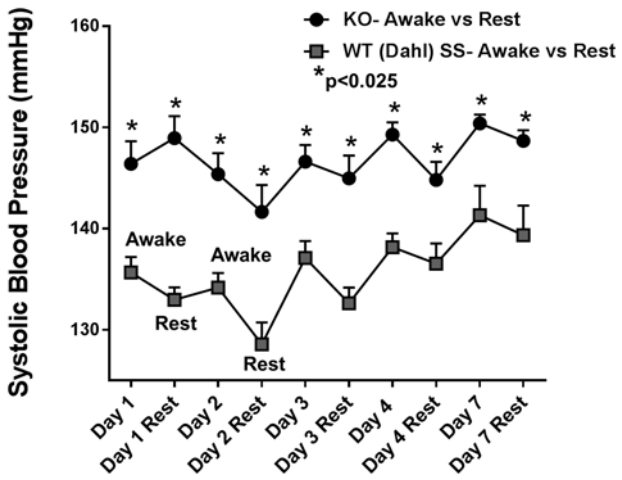


Figure 10. Circadian variation in the systolic blood pressure for the ALMS1 KO and WT (Dahl) SS rats receiving 0.22% of Na Chow. The SBP in the ALMS1 KO rats was significantly higher at baseline (0.22 % Na chow) when measuring the circadian variation in SBP compared to the WT (Dahl) SS rats (KO: 147 ± 1 vs. WT: 136 ± 1 mmHg, p<0.025).

Then we chose to explore the awake vs. rest circadian rhythm of blood pressure measurements (using our 24-hour BP recordings) in order to evaluate the dipping patterns for the ALMS1 KO and WT rats. Blood pressure dipping refers to the difference in blood pressure measurements when going from awake to rest. When measuring the circadian variation in SBP for the circadian cycle (change in SBP from awake to rest) as noted in Figure 10, the ALMS1 KO rats still had a higher SBP in comparison to the WT (Dahl) SS rats (KO: 147 ± 1 vs. WT: 136 ± 1 mmHg, p<0.025). Moreover, when evaluating the change in SBP for the awake-rest cycle there was no difference between the ALMS1 KO and WT rats while on the 0.22% Na diet (Figure 11, KO: 2±1 and WT: 3±0.7 mmHg, p=N.S.).

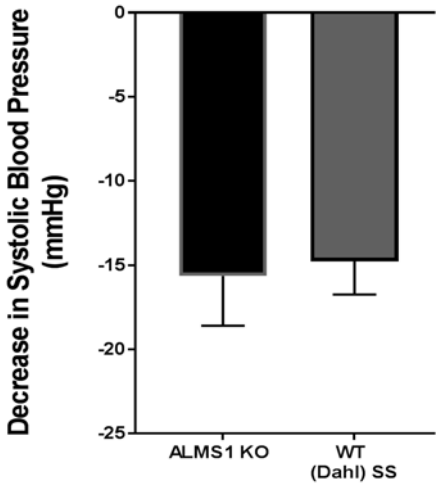


Figure 11. Difference in circadian systolic blood pressure while receiving 0.22% Na chow for the ALMS1 KO and WT (Dahl) SS rats. There was no difference in the SBP from awake to rest during baseline (0.22% Na chow) for either the ALMS1 KO or the WT (Dahl) SS rats (KO: 2 ± 1 vs. WT: 3 ± 0.7 mmHg, p=N.S.)

However, Figure 12 shows that when receiving the 4% Na chow the ALMS1KO rats had a higher SBP compared to the WT rats (KO: 171±2 and WT: 153±1 mmHg, $p<0.025$). Also, Figure 13 shows that the ALMS1 KO rats had a higher increase in circadian variation for SBP compared to the WT rats (KO: 6±0.5 and WT: 5±0.2 mmHg, $p<0.05$). It is in our opinion that the difference between both groups are small (e.g. less than 10 mmHg) and therefore, these data suggest that the ALMS1 KO rats have dipping of their SBP with rest. Overall, our circadian rhythm for BP suggests that the ALMS1 KO rats have intact BP dipping with rest while receiving both the normal salt (0.22% Na) and high salt (4% Na) diets. However, it is important to highlight the greater increases in BP with 4% dietary Na intake in the ALMS1 KO rats, which suggest that these rats have an enhanced salt-sensitivity compared to the WT rats.

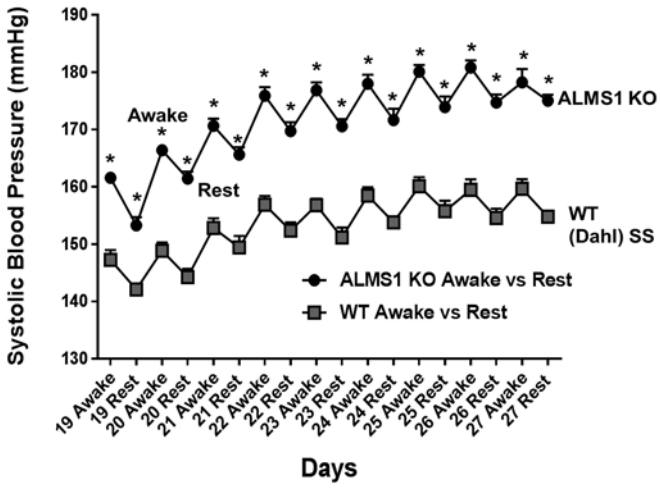


Figure 12. Circadian variation in systolic blood pressure for the ALMS1 KO and WT (Dahl) SS rats while receiving 4% Na chow. The ALMS1 KO rats had a significantly higher SBP when receiving 4% Na chow during the circadian cycle compared to WT (Dahl) SS rats (KO: 171 ± 2 vs. WT: 153 ± 1 mmHg, *$p<0.025$).

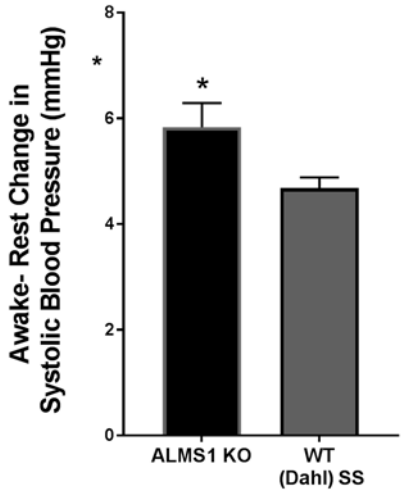


Figure 13. Difference in systolic blood pressure for the ALMS1 KO and WT (Dahl) SS rats while receiving 4% Na chow. There was a significant change in the SBP from awake to rest for the ALMS1 KO rats while receiving the 4% Na chow compared to the WT-SS rats(KO: 6 ± 0.5 and WT: 5 ± 0.2 mmHg, *$p<0.05$).

In addition to the SBP measurements we also evaluated the mean blood pressure (MBP), diastolic blood pressure (DBP) and heart rate (HR) between both groups. Our data show that throughout the experiment the MBP in the awake rats was higher in the ALMS1 KO group compared to the WT group when receiving both the 0.22% (Figure 14, KO: 128.7 ± 2.5 and WT: 121 ± 1.5 mmHg, $p<0.025$) and 4% Na chow (Figure 15, KO: 142 ± 2. Vs. WT: 128 ± 1mmHg, $p<0.025$) in which the ALMS1 KO rats still maintained a higher MBP after receiving bumetanide (Figure 16, KO: 139.1 ± 2.4 and WT: 124.4 ± 2.4 mmHg, $p<0.025$).

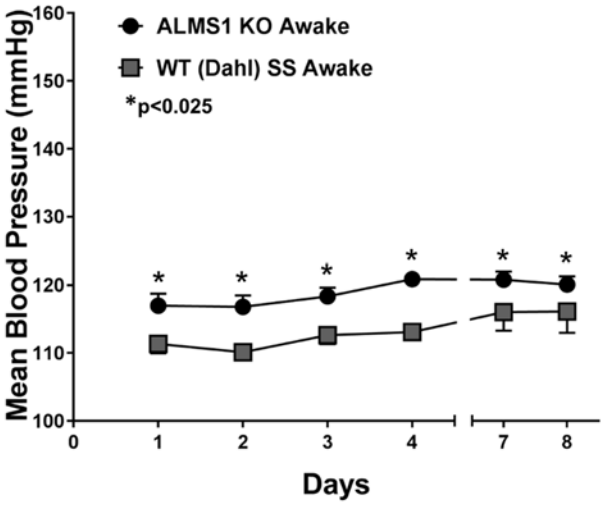


Figure 14. Mean blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 0.22% Na (normal salt) chow using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the normal salt diet (KO: 119 ± 0.8 vs. WT: 113.2 ± 1.0 mmHg, $p<0.025$).

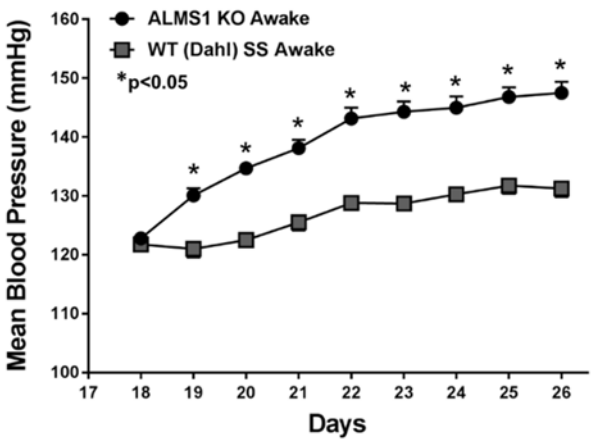


Figure 15. Mean blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the high salt diet (KO: 139.2 ± 2.8 vs. WT: 126.8 ± 1.4 mmHg, $p<0.025$).

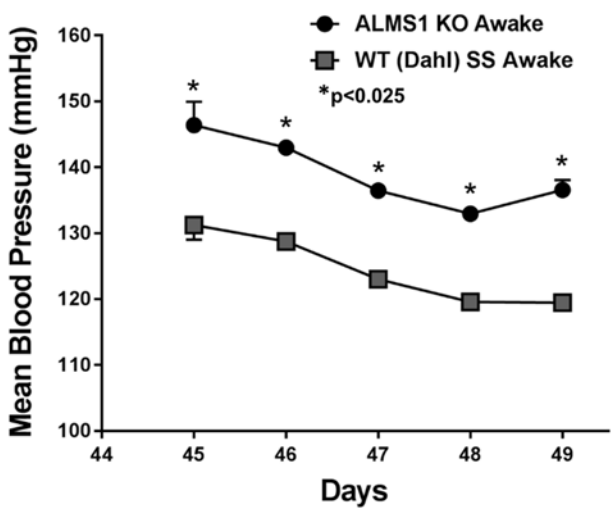


Figure 16. Mean blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow and bumetanide (loop diuretic) using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the high salt diet and bumetanide (KO: 139.1 ± 2.4 vs. WT: 124.4 ± 2.4 mmHg, *p<0.025).

These data resemble that of the SBP for both groups of rats in which the ALMS1 KO rats had a higher MBP with 0.22% and 0.4% Na intake in comparison to the WT (Dahl) SS rats.

Also, the diastolic blood pressure (DBP) was equivalent for both groups with 0.22% Na chow (Figure 17). Yet, the DBP was higher in the ALMS1 KO rats with the 4% Na chow (Figure 18, KO: 113.2 ± 2.3 and WT: 106 ± 1.1 mmHg, p<0.05), and with bumetanide (Figure 19, KO: 112.5 ± 1.9 and WT: 104.4 ± 1.9 mmHg, p<0.05). Ultimately, these data suggest that there is no diastolic hypertension in the ALMS1 KO rats.

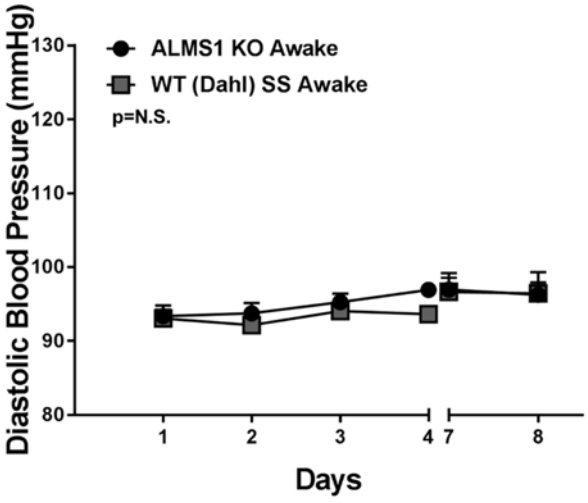


Figure 17. Diastolic blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 0.22% Na (normal salt) chow using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the normal salt diet (KO: 95.42 ± 0.6 vs. WT: 94.33 ± 0.7 mmHg, p=N.S.).

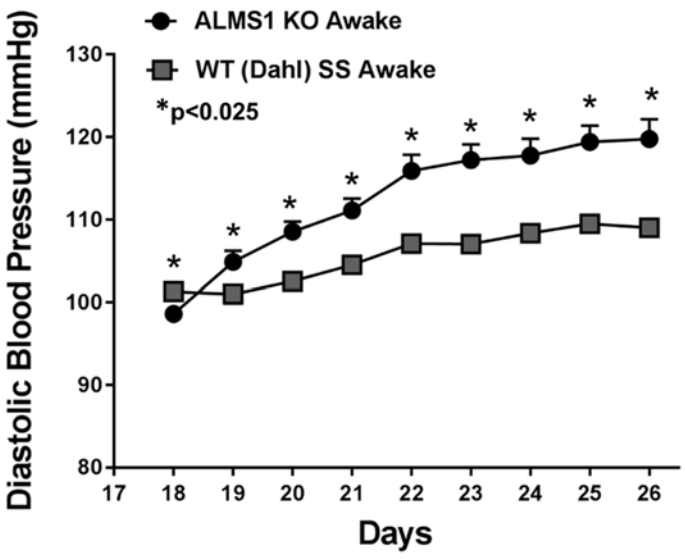
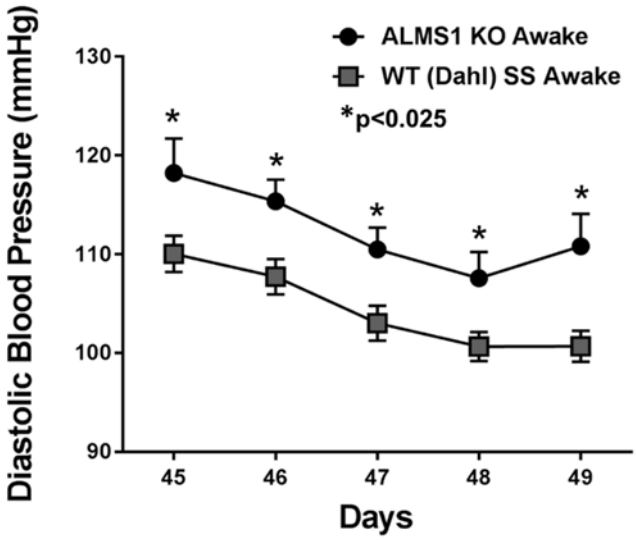


Figure 18. Diastolic blood pressure measurements for the ALMS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow and bumetanide (loop diuretic) using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the high salt diet and bumetanide (KO: 112.6 ± 2.4 vs.105.6 ± 1.11 mmHg, p<0.025).

Figure 19. Diastolic blood pressure measurements for the AMLS1 KO and WT (Dahl) SS rats with 4% Na (high salt) chow and bumetanide (loop diuretic) using invasive radio-telemetry. The invasive blood pressure (BP) telemetry results were obtained in a group of male ALMS1 KO (n=7) and male WT (Dahl) SS (n=7) rats, both at the beginning of the experiment with an average age of 9 weeks old. There was a higher SBP in the awake ALMS1 KO rats compared to the awake WT (Dahl) SS rats with the high salt diet and bumetanide (KO: 112.5 ± 1.9 vs.104.4 ± 1.9 mmHg, p<0.025).



Furthermore, despite the higher systolic hypertension, the ALMS1 KO rats maintained an equivalent heart rate (HR) compared to the WT-SS rats (Figure 20, KO: 331.7 ± 2 vs. WT: 342.8 ± 2.7 bpm, p=N.S.). Only on day 19, the WT rats have a higher HR compared to the ALMS1 KO rats (KO: 335.6 ± 4.6 vs. WT: 366.4 ± 6.2 bpm, p<0.025).

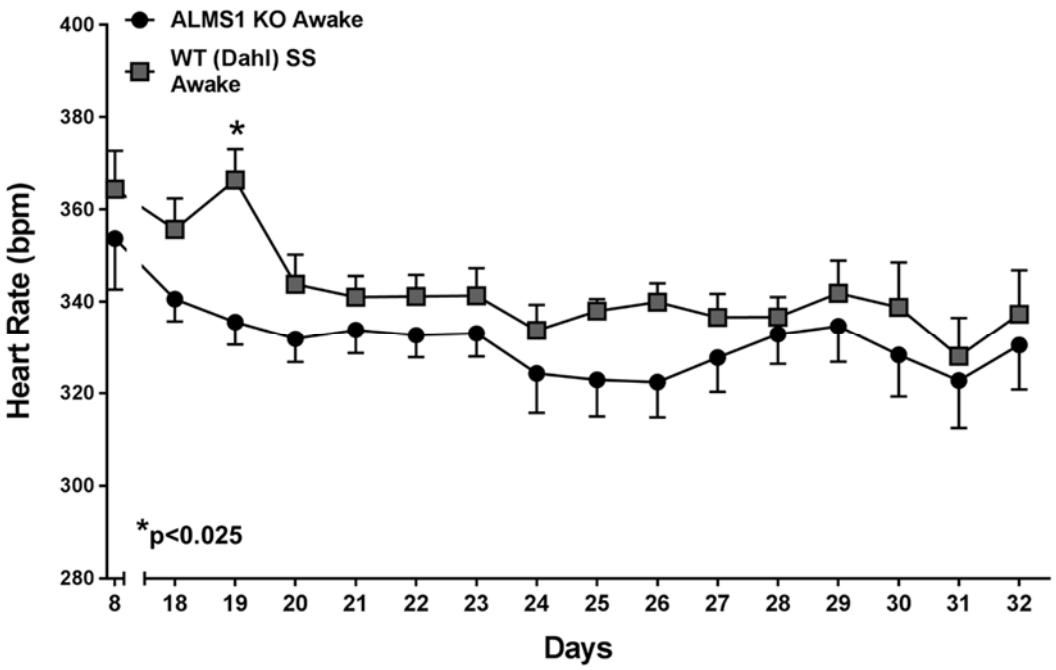


Figure 20. Heart rate measurements were obtained using invasive radio-telemetry for the ALMS1 KO and WT (Dahl) SS rats. ALMS1 KO (n=7) and WT (n=7) rats were approximately 9 week old at the start of this protocol. The awake ALMS1 KO rats had a significantly lower heart rate (HR) in comparison to the awake WT (Dahl) SS rats regardless of the dietary sodium intake (KO: 331.7 ± 2 vs. WT: 342.8 ± 2.7 bpm, p=N.S.).

Overall, these data suggest that the ALMS1 KO rats have a higher MBP and DBP that appears to be exacerbated with increased dietary Na intake compared to the WT rats. Moreover, the HR data shows that both groups have an equivalent HR despite the higher BP in the ALMS1 KO rats. This suggests that other mechanisms including vascular resistance could be elevated in this rat model. However, additional studies would be necessary before concluding the cause of blood pressure elevation in the ALMS1 KO rats.

Non-invasive tail-cuff

We also chose to use tail cuff to explore the blood pressure response to dietary Na intake and diuretics in the ALMS1 KO rats to confirm that the results are equivalent to that for telemetry which supports the use of tail cuff blood pressure measurements for future

experiments. For this experiment we used 8-10 week old male rats were used for this experiment (Figures 21-24). Using noninvasive (restrained) tail cuff blood pressure (BP) measurements we previously found that there was no difference in blood pressure (BP) between the WT and heterozygous (Het) rats (WT: 133±2 and Het: 138±5 mmHg, p=N.S.). The next step was to investigate if there was a difference in BP between these two Dahl SS rats, ALMS1 KO and WT. Figure 21 shows that the ALMS1 KO rats have a higher SBP with both 0.22% and 0.4% Na dietary intake compared the WT (Dahl) SS rats which resembles the results obtained using invasive telemetry.

We found the ALMS1 KO rats to have a higher baseline systolic blood pressure (SBP) on .22% Na chow compared to the WT rats (Figure 21, KO: 136±3 and WT-SS: 125±3 mmHg, p<0.025). After 2 weeks of high Na intake (4% Na chow) the SBP in ALMS1 KO rats increased to 157±2 mmHg, a 21±5 mmHg increase, whereas the SBP in WT rats increased to 140±3 mmHg, 15±6 mmHg increase (ALMS1 KO, p<0.05).

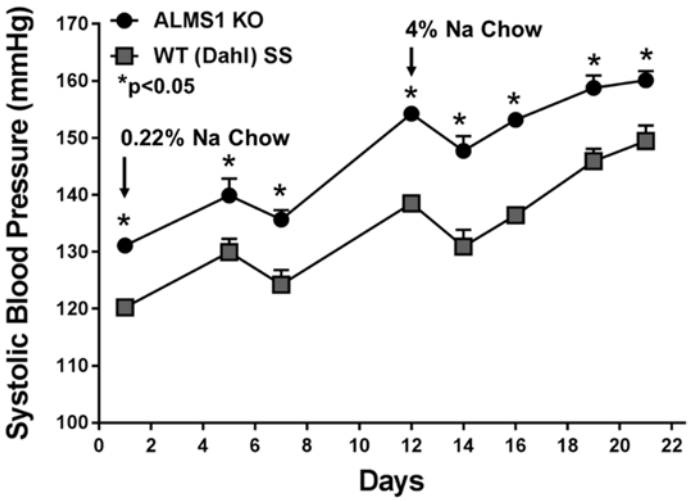


Figure 21. Noninvasive tail-cuff blood pressure measurements were obtained in a group of male ALMS1 KO and male WT rats at various dietary sodium (Na) concentrations. At the beginning of the experiment the rats were approximately 10 weeks old. The ALMS1 KO group had n=6 and the WT group had n=5. We found the ALMS1 KO rats to have a higher baseline systolic blood pressure (SBP) on a normal (.22% Na) chow (KO: 136±3 and WT: 125±3 mmHg, p<0.025 high salt (4% Na) chow compared to the WT rats (KO: 156.8 ± 2.2 vs. WT: 140.2 ± 3.4 mmHg, *p<0.025).

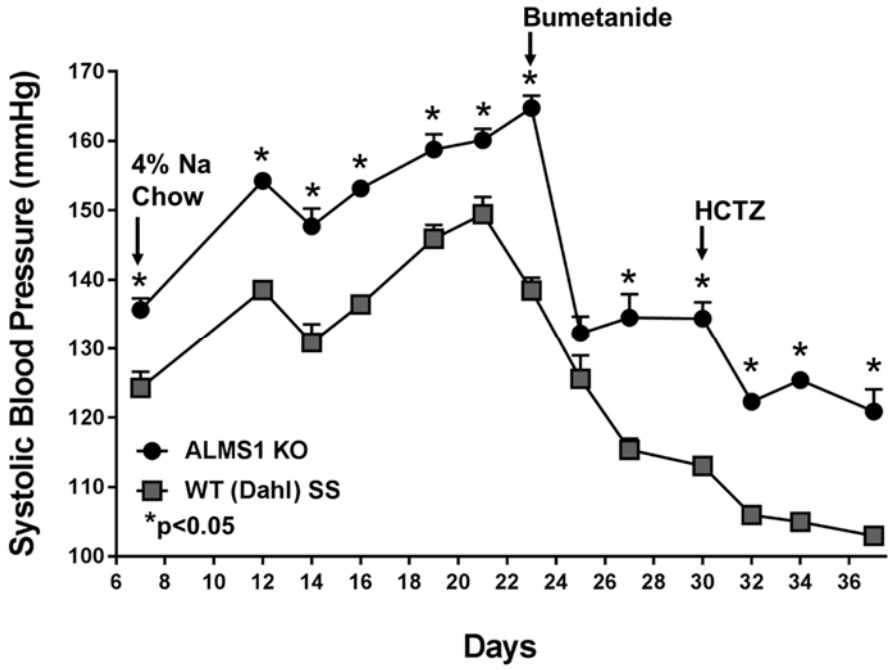


Figure 22. Noninvasive tail-cuff blood pressure measurements were obtained in a group of male ALMS1 KO and male WT rats while receiving 4% Na chow and diuretics. After 2 weeks of high Na intake (4% Na chow) the SBP in ALMS1 KO rats was higher compared to the WT rat (KO: 157±2 vs. WT: 140±3 mmHg, $p<0.05$). The systolic blood pressure remained higher in the ALMS1 KO rats when receiving 4% Na chow and bumetanide alone (KO: 133.7 ± 0.7 vs. WT: 117.7 ± 3.7 mmHg, $p<0.025$) followed by 4% Na chow and hydrochlorothiazide (HCTZ) alone (KO: 122.9 ± 1.4 vs. WT: 104.6 ± 0.9 mmHg, $p<0.025$).

Thus, the SBP was higher in the ALMS1 KO rats fed a high salt (4% Na chow) diet (*$p<0.025$). These data suggest that the ALMS1 KO rats are hypertensive and have an elevation in blood pressure in response to increased dietary Na intake compared to the WT (Dahl) SS rats.

In aims of exploring the hypertension in the ALMS1 KO rats we then gave our rats several diuretics, such as the NKCC2 inhibitor, bumetanide and hydrochlorothiazide (HCTZ) which inhibits the Na/Cl co-transporter (NCC) to further explore the involvement of the thick ascending limb (TAL) and/or distal convoluted tubule, respectively. Figure 23 shows the change in BP for both groups in response to the loop diuretic, bumetanide at a dose of 3mg/kg/d (KO: ─36.89 ± 8 and WT: ─29.24 ± 10 mmHg, p=N.S.). Lastly, after

receiving bumetanide for 1 week (without interruptions) the rats were then given hydrochlorothiazide (HCTZ). Figure 24 shows that both groups had an equivalent diuretic response with this thiazide diuretic, at a dose of 15mg/kg/d (KO: ─50 ± 10 vs. WT: ─50 ± 2 mmHg, p=N.S.).

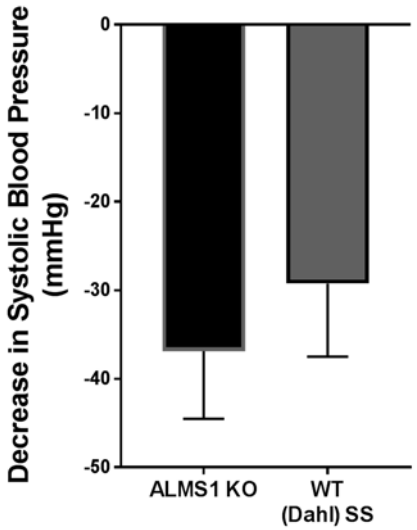


Figure 23. Difference in systolic blood pressure measurements in a group of male ALMS1 KO and male WT (Dahl) SS rats while receiving a 4% Na chow and bumetanide. Both groups had an equivalent diuretic response the loop diuretic, bumetanide (3mg/kg/d) (KO: ─36.89 ± 8 and WT: ─29.24 ± 10 mmHg, p=N.S.).

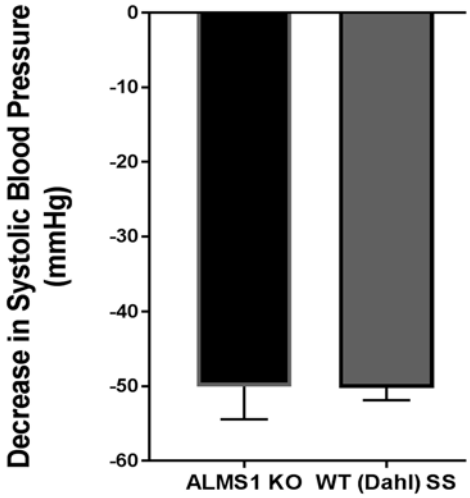


Figure 24. Difference in systolic blood pressure measurements in a group of male ALMS1 KO and male WT (Dahl) SS rats while receiving 4% Na chow and hydrochlorothiazide (HCTZ). Both groups had an equivalent diuretic response the thiazide diuretic, HCTZ (15mg/kg/d) (KO: ─50 ± 10 vs. WT: ─50 ± 2 mmHg, p=N.S.).

Therefore, the BP measurements obtained via the non-invasive tail cuff blood method yield data that was consistent with the data obtained by the invasive radio-telemetry method. The ALMS1 KO rats have a higher SBP in comparison to the WT rats with both 0.22% Na and 4% Na dietary intake. Furthermore, the data suggests that this increase in BP may involve increased renal sodium reabsorption along different nephron segments.

Renal nephron segments involved with the onset of blood pressure (BP) elevation in the ALMS1 knockout (KO) rat

In order to investigate whether the deletion of ALMS1 would interfere with sodium excretion along the nephron segments urine and ion excretion measurements were obtained by metabolic cage experiments. Different diuretics were used to measure the level of natriuresis (along with urinary volume (UV) and urinary osmolality (UOsm) levels) in both groups of rats. The doses for each diuretic was determined based on previous publications as noted in the discussions [29, 55, 109, 119]. As a reminder for this protocol all the rats received 20 grams of regular 0.4% Na chow daily (pair-fed) and during the experiments the rats were fasted while the water intake and urine output was measured.

The baseline urine osmolality was measured in order to determine if there was a difference between the groups for concentrating their urine. When observing the baseline urine osmolality (with food access for 24 hours) the ALMS1 KO rats had a higher urine osmolality compared to the WT rats (Figure 25A, KO: 2457 ± 175 and WT: 1879 ± 177 mOsm/24 hours, $p<0.05$). The baseline urine osmolality results suggest that the ALMS1 KO rats concentrate their urine better than the WT (Dahl) SS rats. This is further supported by the urine volume results which showed there was no difference in the urine volume between the groups of rats (Figure 25B, KO: 6.5 ± 1.5 and WT: 9.2 ± 0.5 ml for 24 hours, p=N.S.).

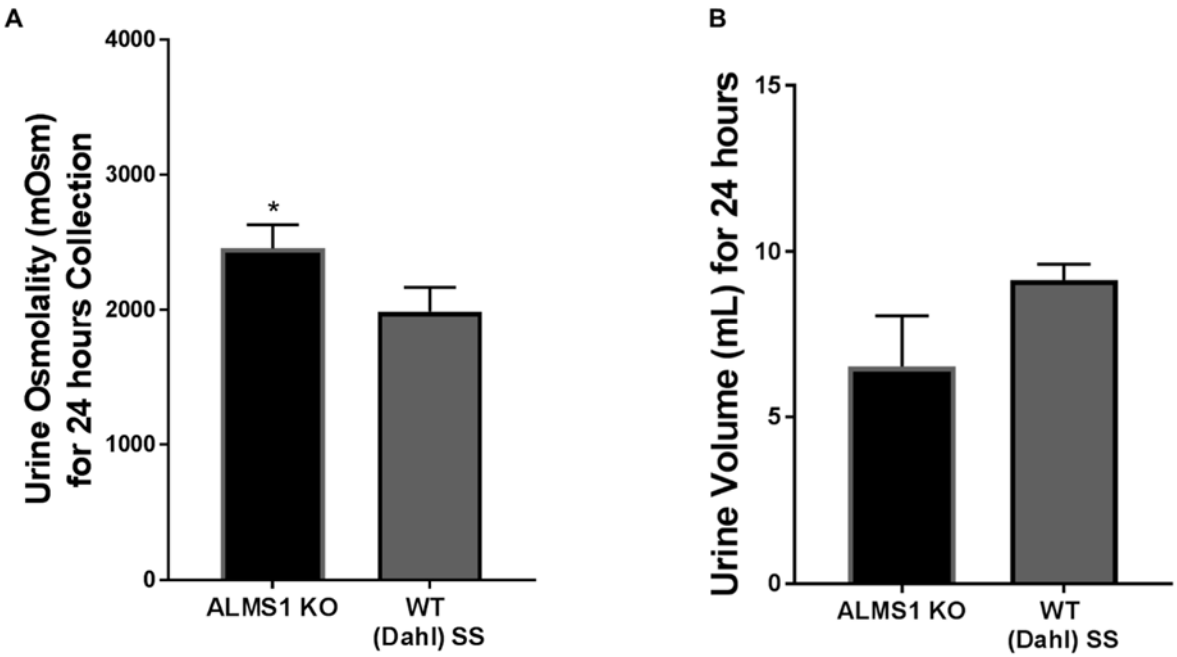


Figure 25. Baseline Urine osmolality (mOsm) and Urine Volume (ml) for the ALMS1 KO and WT (Dahl) SS rats. A) Baseline (BL): The ALMS1 KO rats had a higher cumulative urine osmolality compared to the WT rats (KO: 2457 ± 175 and WT: 1879 ± 177 mOsm per 24 hours, *p<0.05). B) There was no difference in the urine volume between the groups of rats for the 24 hour collection (KO: 6.5 ± 1.5 and WT: 9.2 ± 0.5 ml for 24 hours).

To further explore sodium retention in our rats, we performed the 1% of body weight Acute NaCl Load experiment using metabolic cages to test whether the ALMS1 KO rats have the ability to excrete an acute salt load (Figure 26A-D). After receiving a 1% of body weight (BW) Na load the natriuresis was measured over a period of 12 hours (Figure 26A, 6hr-KO: 409.2 ± 116.5 and WT: 1110 ± 158.9 µmol per hour, p<0.025; 12hr-KO: 190.3 ± 25.7 and WT: 358.6 ± 62.5 µmol per hour, p<0.05). More so, the cumulative natriuretic response to the 1% of body weight Na Load was lower in the ALMS1 KO rats (Figure 26B, KO: 3680 ± 615.5 and WT: 7711 ± 1058 µmol/12 hours, p<0.025).

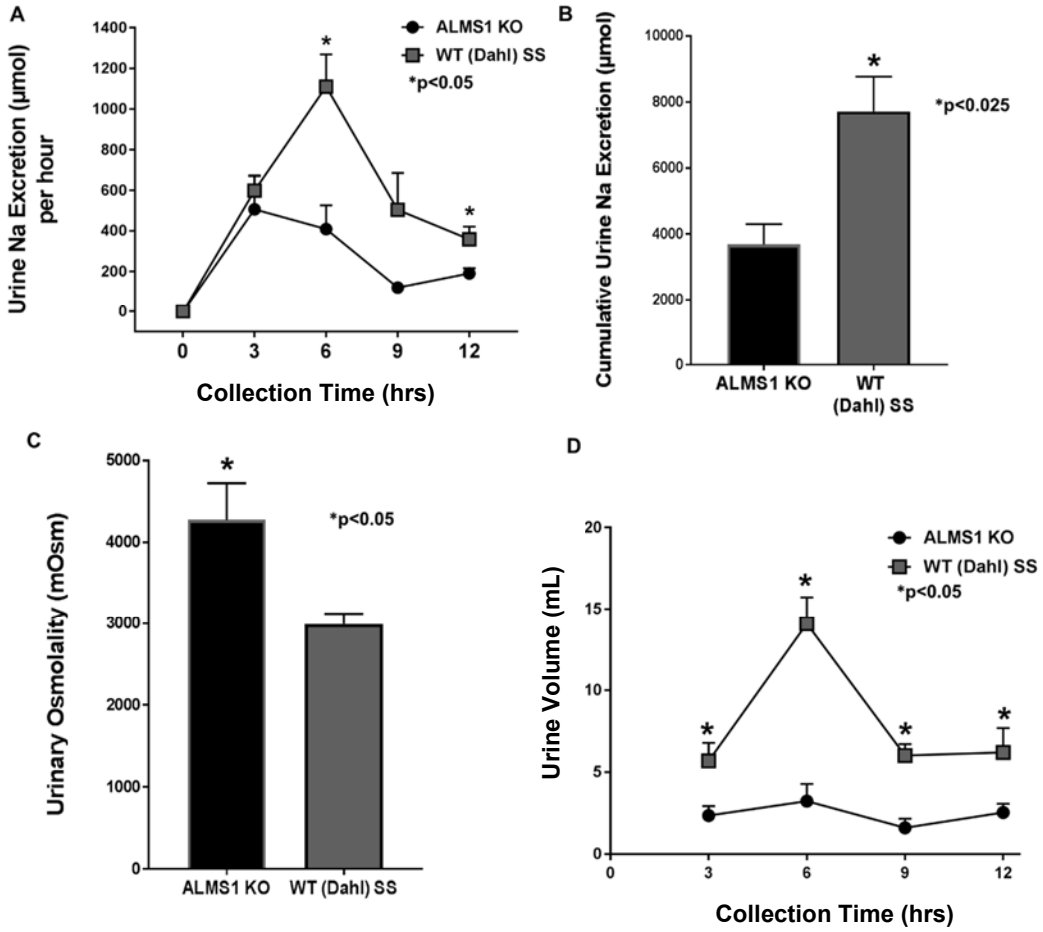


Figure 26. 1% Sodium Chloride (NaCl) load of body weight metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (Dahl) SS (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. Na was administered orally after being mixed in non-fructose containing chocolate frosting. A) 1% NaCl Load: After receiving a 1% of body weight (BW) Na load the ALMS1 KO rats had a lower natriuresis over 12 hours (6hr-KO: 409.2 ± 116.5 and WT: 1110 ± 158.9 µmol per 6 hours, $p<0.025$; 12hr-KO: 190.3 ± 25.7 and WT: 358.6 ± 62.5 µmol per 12 hours, $p<0.05$). B) 1% NaCl Load: The cumulative natriuresis in response to the 1% of BW Na Load was significantly lower in the ALMS1 KO rats (KO: 3680 ± 615.5 and WT: 7711 ± 1058 µmol for 12 hours, $p<0.01$). C) 1% NaCl Load: The ALMS1 KO rats had a higher cumulative urine osmolality compared to the WT rats (KO: 4272 ± 449.8 and WT: 2993 ± 129 mOsm for 12 hours, $p<0.05$). D) 1% NaCl Load: The ALMS1 KO rats had a lower urine output compared to the WT rats (KO: 2.4 ± 0.3 and WT: 8 ± 2. ml for 12 hours, $p<0.05$).

The cumulative urine osmolality was higher in the ALMS1 KO rats (Figure 26C, KO: 4272 ± 449.8 and WT: 2993 ± 129 mOsm/12 hours, $p<0.05$) while the urine volume was lower (Figure 26D, KO: 2.4 ± 0.3 ml and WT: 8 ± 2 ml for 12 hours, $p<0.05$). These results suggest that the ALMS1 KO rats have a decreased ability to excrete an acute 1% of body weight Na load compared to the WT rats.

Next, when giving the loop diuretic, bumetanide (inhibits NKCC2) at the dose 20mg/kg, the ALMS1KO rats had a higher urinary natriuresis compared to the WT rats (Figure 27A, 4hr-KO: 195.5 ± 51.0 and WT: 31 ± 11 µmol per hour, p<0.025).

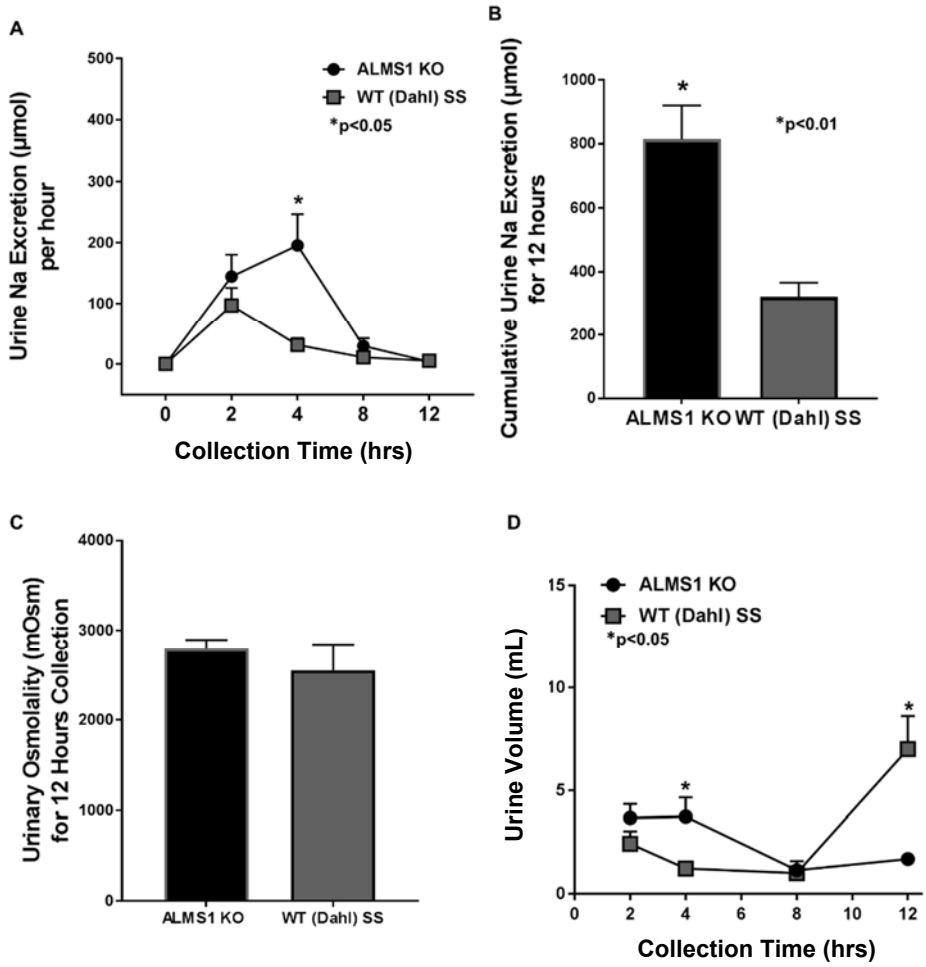


Figure 27. Bumetanide metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (Dahl) SS (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. Bumetanide was administered orally after being mixed in non-fructose containing chocolate frosting. A) Bumetanide: After receiving bumetanide at a dose of 20 mg/kg the ALMS1 KO rats had a higher natriuretic response compared to the WT rats (4hr-KO: 195.5 ± 51.0 and WT: 31 ± 11 µmol per 4 hours, p<0.025). B) Bumetanide: The ALMS1 KO rats had a greater cumulative natriuretic response with bumetanide compared to the WT rats (KO: 984.2 ± 127.5 and WT: 522.6 ± 67.47 µmol per 12 hours, p< 0.01). C) Bumetanide: There was no difference in the urine osmolality between both groups (KO: 2794 ± 100.6 and WT: 2553 ± 290.8 mOsm per 12 hours, p=.4505). D) Bumetanide: ALMS1 KO rats had a higher urine volume at the 4 hour (KO: 3.7 ± 0.9 and WT: 1.2 ± 0.3 ml per hour, p<0.05) and 12 hours (KO: 1.7 ± 0.2 and WT: 6.995 ± 1.611 ml per hour, p<0.025) collection times.

Also, the ALMS1 KO rats had a greater cumulative natriuretic response with bumetanide (Figure 27B, KO: 984.2 ± 127.5 and WT: 522.6 ± 67.5 µmol/12 hours, $p<0.025$). Yet, there was no difference in the urine osmolality between both groups (Figure 27C, KO: 2794 ± 100.6 vs. WT: 2553 ± 290.8 mOsm/12 hours, p=N.S.). Also, the ALMS1 KO rats had a higher urine volume at the 4 hour (KO: 3.7 ± 0.9 and WT: 1.2 ± 0.3 ml per hour, $p<0.05$) and 12 hours (KO: 1.7 ± 0.2 and WT: 6.995 ± 1.611 ml per hour, $p<0.025$) collection times. Overall, these results suggest that there is increased renal Na reabsorption along the thick ascending limb (TAL) via the NKCC2 cotransporter.

In aims of determining the cause of the enhanced renal Na absorption in the ALMS1 KO rats we then decided to explore Na reabsorption along the proximal nephron. We used the diuretic, acetazolamide, which is a carbonic anhydrase inhibitor, at a dose of 50mg/kg. The ALMS1 KO rats had a lower natriuretic response compared to the WT rats (Figure 28A, 2hr-(KO: 288.7 ± 55.6and WT: 582.4 ± 41.76 µmol/12 hours, $p<0.025$). Furthermore, the ALMS1 KO rats had a lower cumulative natriuretic response with acetazolamide compared to the WT rats (Figure 28B, KO: 635.4 ± 105.6 and WT: 997.7 ± 39.54 µmol/12 hours, $p<0.025$). Then the cumulative urine osmolality was higher in the ALMS1 KO rats (Figure 28C, KO: 2634 ± 67.05 and WT: 2031 ± 161.3 mOsm/12 hours, $p<0.025$); while the urine volume (Figure 28D) was higher in the WT rats at the 2 hour (KO: 3.6 ± 0.5 and WT: 6.2 ± 0.2 ml per hour, $p<0.025$), 4 hour (KO: 0.8 ± 0.04 and WT: 1.9 ± 0.23 ml per hour, $p<0.025$) and 12 hour (KO: 2.2 ± 0.46 and WT: 4.6 ± 0.49 ml per hour, $p<0.025$) collection times. The results for acetazolamide suggest that the proximal tubule may not be involved in enhanced renal Na reabsorption in the ALMS1 KO rats.

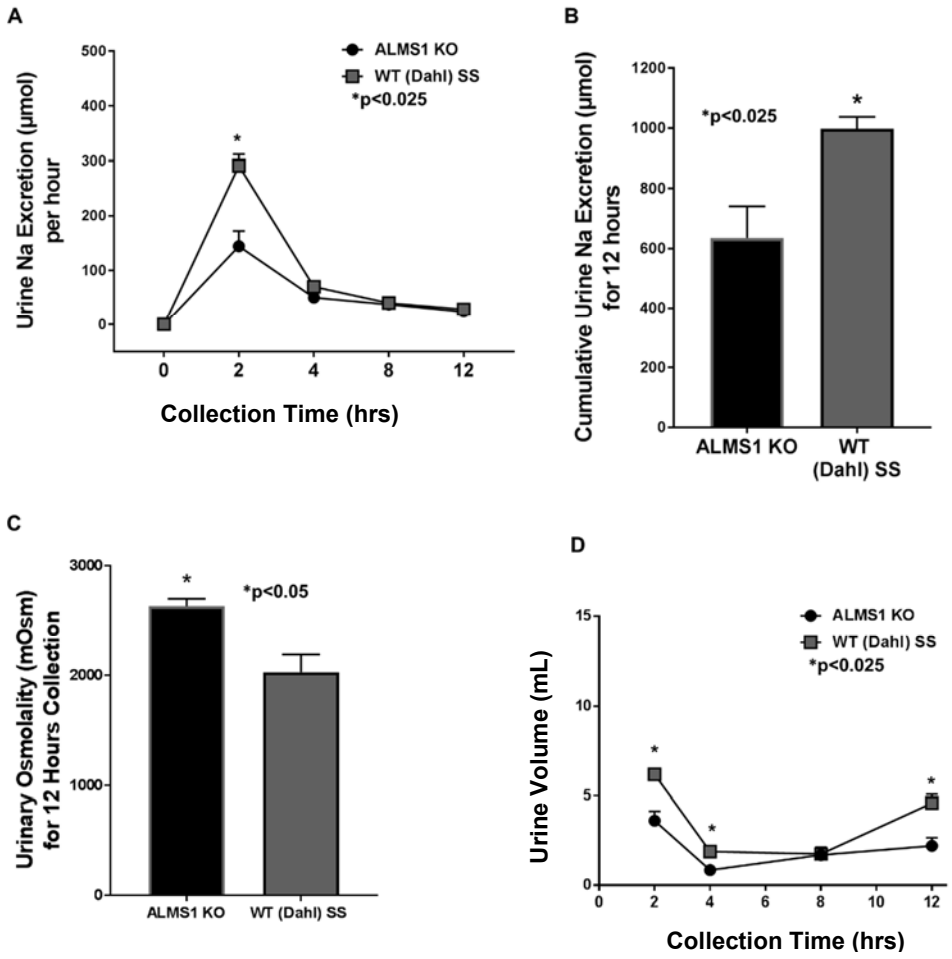


Figure 28. Acetazolamide metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (Dahl) SS (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. Acetazolamide was administered orally after being mixed in non-fructose containing chocolate frosting. A) Acetazolamide: After receiving acetazolamide at a dose of 50 mg/kg the ALMS1 KO rats had a lower natriuretic response compared to the WT rats (2hr-KO: 288.7 ± 55.6and WT: 582.4 ± 41.76 µmol per 2 hours, p<0.025). B) Acetazolamide: The ALMS1 KO rats had a lower cumulative natriuretic response with acetazolamide compared to the WT rats (KO: 635.4 ± 105.6 and WT: 997.7 ± 39.54 µmol per 12 hours, p<0.01). C) Acetazolamide: The ALMS1 KO rats had a higher cumulative urine osmolality compared to the WT rats (KO: 2634 ± 67.05 and WT: 2031 ± 161.3 mOsm per 12 hours, p<0.01). D) Acetazolamide: The ALMS1 KO rats had a lower urine volume at the 2 hour (KO: 3.6 ± 0.5 and WT: 6.2 ± 0.2 ml per hour, p<0.025), 4 hour (KO: 0.8 ± 0.04 and WT: 1.9 ± 0.23 ml per hour, p<0.025) and 12 hour (KO: 2.2 ± 0.46 and WT: 4.6 ± 0.49 ml per hour, p<0.025) collection times.

After the results did not support the concept of proximal tubule involvement we then decided to explore the distal nephron segments. We used the diuretic hydrochlorothiazide (HCTZ) which inhibits the Na-Cl cotransporter (NCC) in the distal convoluted tubule.

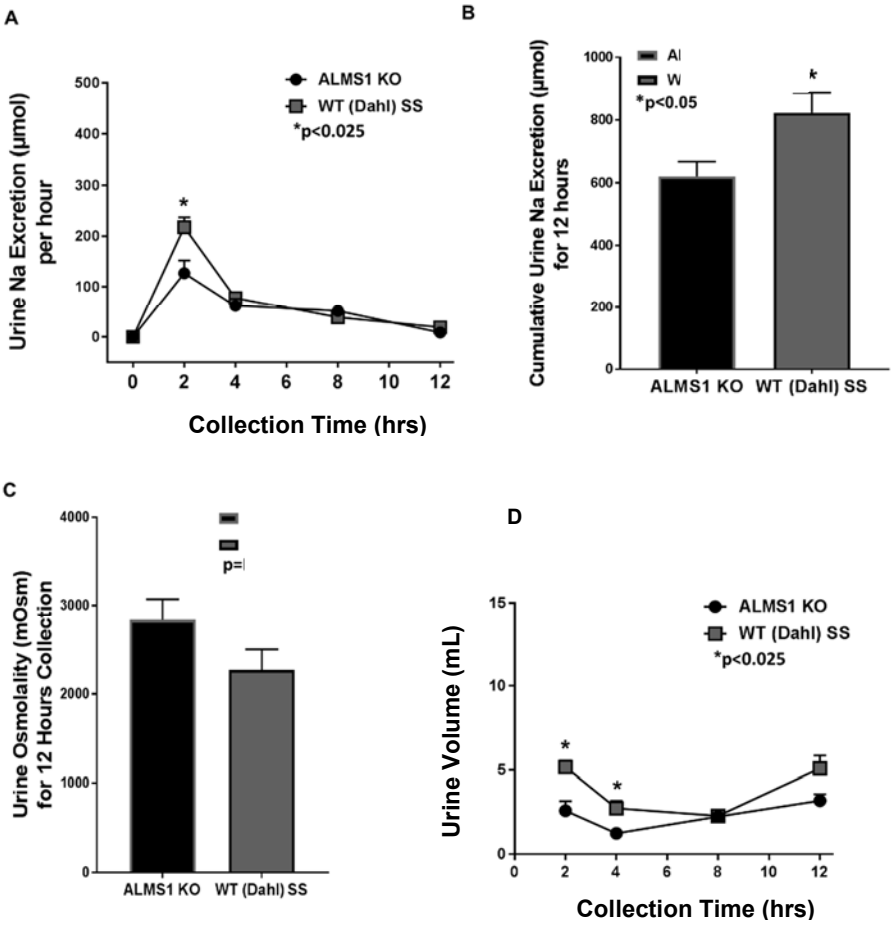


Figure 29. Hydrochlorothiazide (HCTZ) metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (Dahl) SS (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. HCTZ was administered orally after being mixed in non-fructose containing chocolate frosting. A) HCTZ: After receiving HCTZ at a dose of 200 mg/kg the ALMS1 KO rats had a lower natriuretic response compared to the WT rats (2hr-KO: 144.4 ± 27.8 and WT: 291.2 ± 20.9 µmol per 2 hours, p<0.025; 12hr- KO: 35.18 ± 4.221 and WT: 75.7 ± 2.18 µmol per 12 hours, p<0.025). B) HCTZ: The ALMS1 KO rats had a lower cumulative natriuretic response with HCTZ compared to the WT rats (KO: 620.5 ± 47.95 and WT: 822.6 ± 64.74 µmol, per 12 hours p<0.05). C) HCTZ: There was no difference in the urine osmolality between both groups (KO: 2833 ± 240 and WT: 2270 ± 230.6 mOsm per 12 hours, p=.1217). D) HCTZ: The ALMS1 KO rats had a lower urine output compared to the WT rats at the 2 hour (KO: 3.6 ± 0.52 and WT: 6.2 ± 0.21 ml per hour, p<0.025) and 4 hour (KO: 0.9 ± 0.04 and WT: 1.9 ± 0.23 ml per hour, p<0.025) collection times.

The ALMS1 KO rats were found to have a lower natriuresis with HCTZ (200mg/kg) compared to the WT rats (Figure 29A, 2hr-KO: 144.4 ± 27.8 and WT: 291.2 ± 20.9 µmol per hour, p<0.025; 12hr- KO: 35.18 ± 4.221 and WT: 75.7 ± 2.18 µmol per 12 hours, p<0.025). Plus, the ALMS1 KO rats had a lower cumulative natriuretic response with

HCTZ compared to the WT rats (Figure 29B, KO: 620.5 ± 47.95 and WT: 822.6 ± 64.74 mOsm/12 hours, $p<0.05$). Regarding the urine osmolality in response to HCTZ, there appears to be no difference between the groups (Figure 29C, KO: 2833 ± 240 and WT: 2270 ± 230.6 mOsm/12 hours, N.S.). Also, as shown in Figure 29D, the ALMS1 KO rats had a lower urine output compared to the WT rats at the 2 hour (KO: 3.6 ± 0.52 and WT: 6.2 ± 0.21 ml per hour, $p<0.025$) and 4 hour (KO: 0.9 ± 0.04 and WT: 1.9 ± 0.23 ml per hour, $p<0.025$) collection times. These results suggest that the NCC cotransporter along the distal convoluted tubule is not involved in higher Na reabsorption in the ALMS1 KO rats. Then we explored if the collecting duct for involvement by using the diuretics, benzamil and amiloride, which both inhibit the epithelial-Na channel (ENaC) located in the collecting duct.

Next, the results for benzamil (10mg/kg) showed the ALMS1 KO rats to have a lower natriuresis compared to the WT rats (Figure 30A, 4hr-KO: 44.7 ± 15.21 and WT: 143.7 ± 7.6 µmol per 4 hours, $p<0.025$; 12hr- KO: 13.41 ± 1.4 and WT: 26.21 ± 1.4 µmol per 12 hours, $p<0.025$). The ALMS1 KO rats had a lower cumulative natriuretic response with benzamil compared to the WT rats as well (Figure 30B, KO: 520.2 ± 97.23 and WT: 958.2 ± 39.17 µmol per 12 hours, $p<0.01$).

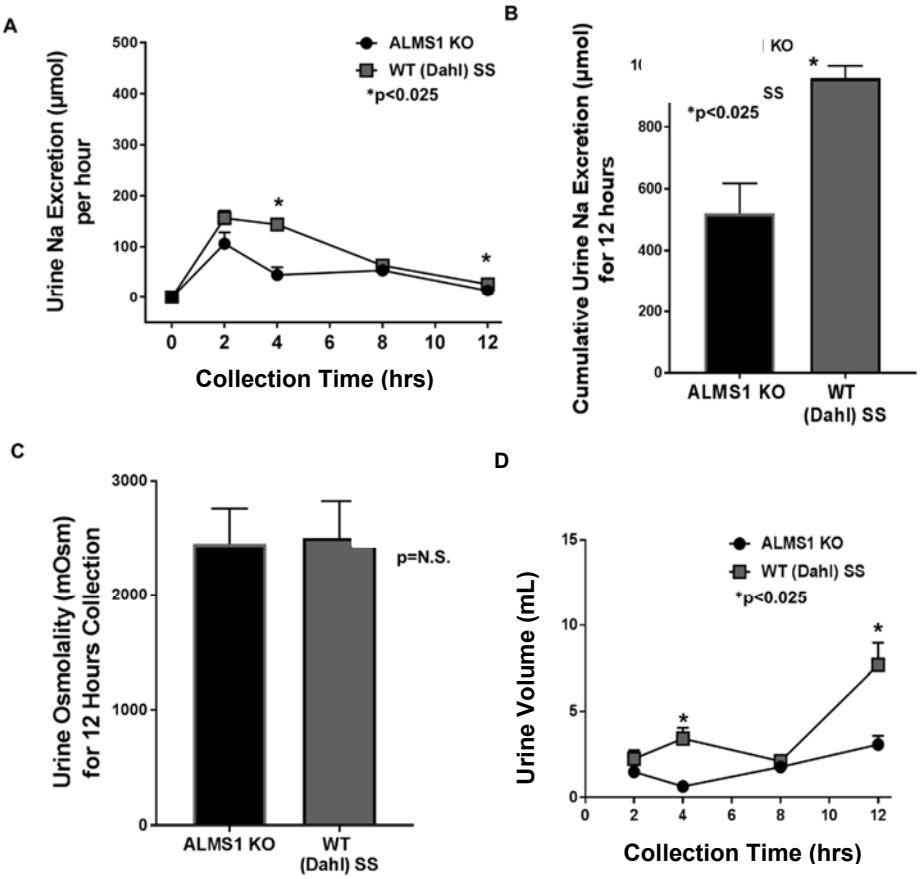


Figure 30. Benzamil metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. Benzamil was administered orally after being mixed in non-fructose containing chocolate frosting. A) Benzamil: After receiving benzamil at a dose of 10 mg/kg the ALMS1 KO rats had a lower natriuretic response compared to the WT rats (4hr-KO: 44.7 ± 15.21 and WT: 143.7 ± 7.6 µmol per 4 hours, p<0.025; 12hr- KO: 13.41 ± 1.4 and WT: 26.21 ± 1.4 µmol per 12 hours, p<0.025). B) Benzamil: The ALMS1 KO rats had a lower cumulative natriuretic response with benzamil compared to the WT rats (KO: 520.2 ± 97.23 and WT: 958.2 ± 39.17 µmol per 12 hours, p<0.025). C) Benzamil: There was no difference in the urine osmolality between both groups (KO: 2451 ± 309.2 and WT: 2503 ± 322.3 mOsm per 12 hours, p=.9111). D) Benzamil: The urine volume was lower in the ALMS1 KO rats at the 4 hour (KO: 0.6 ± 0.23 and WT: 3.4 ± 0.63 ml per hour, p<0.025) and 12 hour (KO: 3.1 ± 0.5 and WT: 7.7 ± 1.3 ml per hour, p<0.025) collection times.

And there does not appear to be a difference in the urine osmolality between both groups (Figure 30C, KO: 2451 ± 309.2 and WT: 2503 ± 322.3 mOsm per 12 hours, N.S.); yet, the urine volume was lower in the ALMS1 KO rats at the 4 hour (KO: 0.6 ± 0.23 and WT: 3.4 ± 0.63 ml per hour, p<0.025) and 12 hour (KO: 3.1 ± 0.5 and WT: 7.7 ± 1.3 ml per hour, p<0.025) collection times.

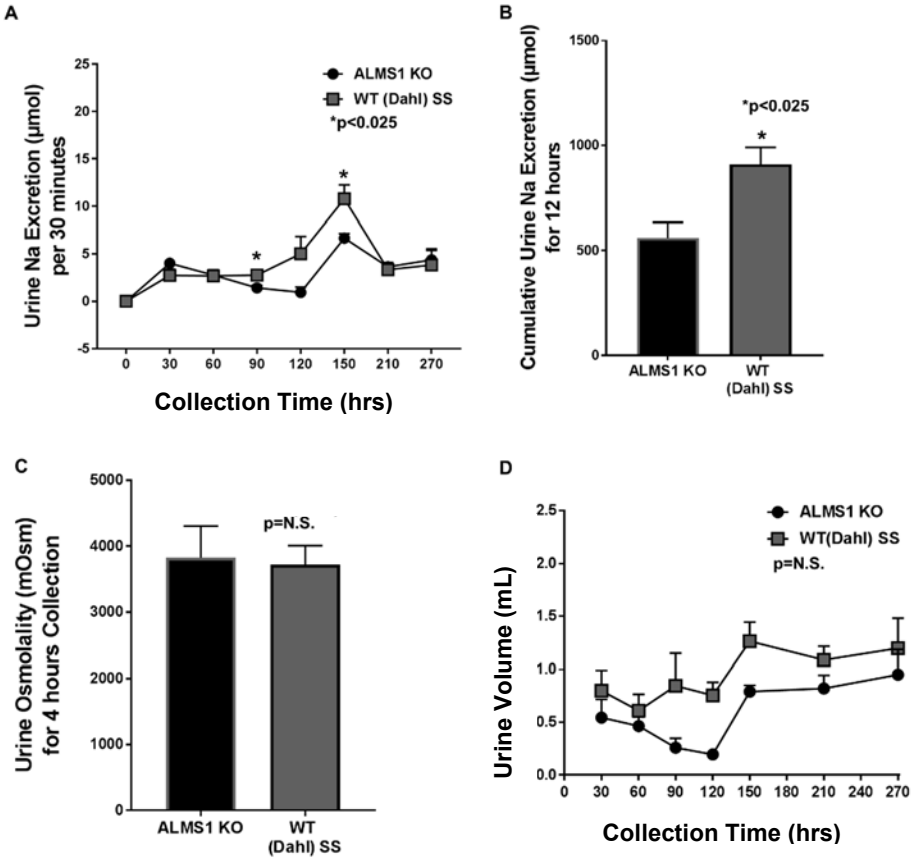


Figure 31. Amiloride metabolic cage experiment for urine and ion excretion measurements. Each group consisted of male KO (n=6) and male WT (n=6) with an average age of 8-9 weeks old at the beginning of the experiment. Amiloride was administered orally after being mixed in non-fructose containing chocolate frosting. A) Amiloride: After receiving amiloride at a dose of 20 mg/kg the ALMS1 KO rats had a lower natriuretic response compared to the WT rats (90min-KO: 1.43 ± 0.28 and WT: 2.778 ± 0.2 µmol per 90 minutes, $p<0.025$; 150min- KO: 6.7 ± 0.5 and WT: 10.8 ± 1.5 µmol per 150 minutes, $p<0.025$). B) Amiloride: The ALMS1 KO rats had a lower cumulative natriuretic response with amiloride compared to the WT rats (KO: 561.1 ± 72.65 and WT: 910.2 ± 80.23 µmol per 270 minutes, $p<0.01$). C) Amiloride: There was no difference in the urine osmolality between both groups (KO: 3824 ± 481.2 and WT: 3717 ± 290.5 mOsm per 270 minutes, $p=.8517$). D) Amiloride: There was no difference in urine volume between both groups in response to amiloride.

Lastly, the results for amiloride (20mg/kg) also showed the ALMS1 KO rats have a lower natriuresis compared to the WT (Figure 31A, 90min-KO: 1.43 ± 0.28 and WT: 2.778 ± 0.2 µmol per 90 minutes, $p<0.025$; 150min- KO: 6.7 ± 0.5 and WT: 10.8 ± 1.5 µmol per 150 minutes, $p<0.025$). Then our data showed that the ALMS1 KO rats had a lower cumulative natriuretic response with amiloride as well (Figure 31B, KO: 561.1 ± 72.65 and WT: 910.2 ± 80.23 µmol per 270 minutes, $p<0.025$). Also, there was no difference in the

urine osmolality between both groups (Figure 31C, KO: 3824 ± 481.2 vs. WT: 3717 ± 290.5 mOsm per 270 minutes, p=N.S.). Finally, Figure 31D shows that the ALMS1 KO rats have an equivalent urine volume to the WT rats. Overall, the data for both benzamil and amiloride show that in absence if this inhibition there is no enhanced ENaC activity in the ALMS1 KO rats. These results suggest there is no increased Na reabsorption along the collecting duct via ENaC in response to ALMS1 deletion in rats.

Therefore, the elevation in BP seen in the ALMS1 KO rats appear to be exacerbated by increased dietary Na intake that is likely mediated by enhanced NaCl reabsorption in the thick ascending limb (TAL) via increased NKCC2 activity.

Body weight

For all the protocols we monitored the body weight (BW) for both groups of rats. This allowed us to monitor for change in body habitus which is of most importance given the ALMS1 KO rats are hyperphagic and visually appear to be bigger than the WT rats. However, given that we did not evaluate the plasma volume we cannot exclude this as a contributor to the increase in body weight in the ALMS1 KO rats. However, the body weight (BW) measurements from the previous experiments discussed were obtained in both groups ranging from 6-20 weeks of age.

Figure 32A shows that the ALMS1 KO rats had a greater BW by 14 weeks of age in comparison to the WT rats (KO: 479 ± 5.323 and WT: 396 ± 4.024 g, $p<0.025$) during the telemetry protocol. Then Figure 32B shows the BW during the non-invasive tail-cuff BP experiment. Here the ALMS1 KO rats began to separate in size from the WT rats at 9 weeks of age. However, the ALMS1 KO rats achieved a larger BW at 12-16 weeks of age (KO: 425.4 ± 9 and WT: 353.7 ± 6 g, $p<0.025$. Overall, by 4 months old, the ALMS1 KO rats had a larger body weight compared to the WT rats.

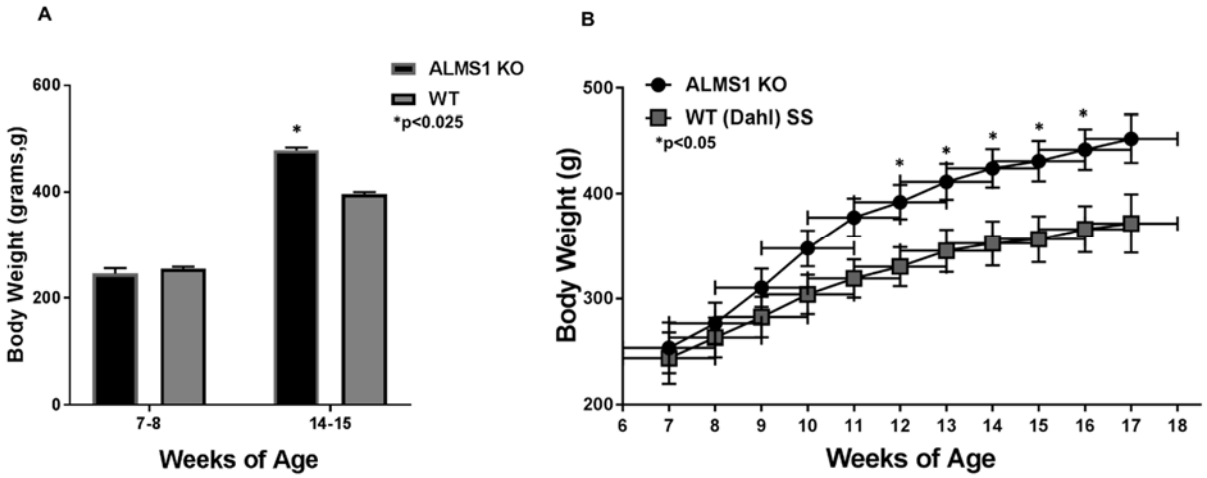


Figure 32.The body weight (BW) measurements obtained for the ALMS1 KO rats compared to the WT (Dahl) SS rats ranging in age from 6-20 weeks old for all experiments. A) For the invasive radio-telemetry blood pressure (BP) experiment two body weight measurements were obtained and ALMS1 KO rats had a greater BW by 14 weeks of age in comparison to the WT rats (KO: 479 ± 5.323 and WT: 396 ± 4.024 g, $p<0.025$). B) During the non-invasive tail cuff BP experiment the ALMS1 KO rats begin to separate from the WT rat at 9 weeks of age. However, the ALMS1 KO rats achieved a larger body weight from 12-16 weeks of age (KO: 425.4 ± 9 and WT: 353.7 ± 6 g, $p<0.025$).

Summary

Our research demonstrates that at various dietary Na intakes, the ALMS1 KO rats have a higher systolic blood pressure (SBP) compared to the WT (Dahl) SS rats. Furthermore, the ALMS1 KO rats have a greater increase in BP with higher dietary Na intake compared to the WT (Dahl) SS rats. And also, our results show that the ALMS1 KO rats have a higher body weight. Our laboratory obtained BP measurements using both the noninvasive tail cuff plethysmography and the invasive radio-telemetry BP measurements in which the results were consistent. Our data strongly suggests that the hypertension observed in the ALMS1 KO rat is influenced by increased activity of the Na-K-2Cl cotransporter (termed NKCC2) which enhances NaCl reabsorption along the thick ascending limb (TAL) of the Loop of Henle.

However, our radio-telemetry telemetry data when administering oral bumetanide (loop diuretic) revealed that other mechanisms may be contributing to the elevation of BP

in the ALMS1 KO rats. So we explored the involvement of different nephron segments in blood pressure elevation and salt sensitivity in the ALMS1 KO rats. Using a metabolic cage protocol we found that bumetanide (NKCC2 inhibitor) yield data that suggest increased NaCl reabsorption along the TAL in the absence of this inhibition in the ALMS1 KO rats. However, the proximal tubule, distal convoluted tubule and collecting duct did not appear to be involved in decreased Na urinary excretion in this rat model. Nonetheless, obesity is a strong risk factor for hypertension [17, 37, 57]. As shown in Figure 32, the ALMS1 KO rats have an increase in body weight with growth which led us to explore obesity in relation to hypertension in this rat model.

CHAPTER 3: HYPERTENSION PRECEDES OBESITY AND METABOLIC SYNDROME IN THE ALMS1 (ALSTRÖM SYNDROME 1) KNOCKOUT RAT

Introduction

Metabolic Syndrome is a medical condition consisting of obesity, hypertension, hyperglycemia, and hypertriglyceridemia [37, 913]. Metabolic syndrome is also a risk factor for cardiovascular disease and type 2 diabetes [37, 93]. In the U.S.1 of 4 adults have metabolic syndrome and the prevalence of metabolic syndrome increases with age [37, 93]. Furthermore, this medical condition has been shown to be influenced by race and ethnicity in which certain groups have a higher risk of developing metabolic syndrome [37, 93]. More specifically, obesity is linked to several other medical comorbidities such as type 2 diabetes, cardiovascular heart disease and cancer [37]. This is of particular interest to our laboratory because as noted previously, we collected data that suggests the ALMS1 KO rats develop progressive obesity which is also a characteristic noted in both the human population diagnosed with Alström Syndrome (ALMS), as well as the mutant ALMS1 mouse model [9, 89-91].

Currently, both clinical and laboratory communities are conducting research on ALMS1 to determine the underlying causes for the abnormal phenotypic characteristics seen in patients with ALMS [82 and 88-89]. Based on extensive clinical research conducted by Marshall and colleagues, it is known that ALMS patients develop early hyperinsulinemia and childhood obesity that typically converts to a lower body lipodystrophic pattern [62]. Moreover, there are scientific researchers that have shown ALMS1 playing a role in cilium formation and/or function [31]. This is of most importance because it has been shown to influence the renal impairment, cardiomyocyte proliferation, and alterations in recycling endosome pathways that could influence protein trafficking in

response to ALMS1 deficiency [32, 52 and 113].

In our laboratory we are currently exploring the underlying causes of blood pressure elevation in the ALMS1 KO rats. Given that several studies have shown that obesity, either genetic or induced by diet, increased blood pressure we wanted to determine if the hypertension in the ALMS1 KO rat is a consequence of obesity. To determine the non-renal underlying influences for the elevation of blood pressure in the ALMS1 KO rats, we tested whether a caloric restriction would decrease body weight (BW) and this in turn would lower blood pressure [37, 93, 97].

Methods

Body weight measurements

As noted previously in the methods section of chapter 2.

Blood pressure measurements

Systolic blood pressure (SBP) was measured using Kent Scientific corporation non-invasive tail-cuff plethysmograph as previously described in chapter 2.

Fasting glucose tolerance testing (GTT)

The GTT was performed every 4 weeks after the rats were fasted overnight for 16 hours followed by an intraperitoneal (i.p.) injection of glucose (2 g/100kg BW). Blood samples were taken from the tip of the tail after performing a single tail snip to collect capillary blood and glucose was measured using the MooreBrand TRUE METRIX PRO Professional glucose meter and test strips (Farmington, CT 06032-4066) at 0 (fasting), 15, 30, 60, 90 and 120 minute intervals. Rats were kept housed separately in their cages with free access to regular water during the experiment. Obtained data were used in GraphPad PRISM to calculate the area under the glucose curves (AUC). Statistical analysis for AUC was done using one-way ANOVA when comparing two means and 2-way

ANOVA when comparing more than 2 means.

Random (Non-fasting) serum insulin collection

The serum insulin is collected randomly (*random means non-fasting or collected at any given time of the day*). During collection the rats were anesthetized using isoflurane while approximately 0.5 ml of blood was collected from the tail vein. The blood was left to clot over 10-15 minutes before centrifuging at 1000 rpm for 10 minutes. The serum was then collected and put into a -4°F freezer until it was analyzed using the insulin enzyme immunoassay kit (#A05105.96 wells) by Spi Bio (Bertin Pharma, France).

Random (non-fasting) serum leptin collection

The serum leptin was collected randomly (*random means non-fasting or collected at any given time of the day*). During collection the rats were sedated using isoflurane while approximately 0.5 ml of blood was collected from the tail vein. The blood was left to clot over 10-15 minutes before centrifuging at 1000 rpm for 10 minutes. The serum was then collected and put into a -4°F freezer until it was analyzed using the mouse/rat leptin EIA enzyme immunoassay kit (#A05176.96 wells) by spi bio (Bertin Pharma, France).

Metabolic cage urine collection

Urine and ion excretion measurements were performed and Na+/K+ and osmolality measurements were analyzed as previously described in chapter 2.

Kidney weight collections

In order to correctly determine the renal function the kidney weights were obtained. The kidneys were surgically removed and then weighed.

GFR measurements

Glomerular filtration rate measurements were performed in conscious rats according to the methods described T. Rieg, et al. [106]. This method is based on

calculating the clearance of fluorescein isothiocyanate (FITC)-labeled inulin clearance using a two-compartment model after a single bolus tail vein injection. FITC-inulin (TdB Consultancy, Sweden) was prepared in sterile saline and dialyzed with 3.5 kDa membrane (Millipore 71508). Rats were transiently anesthetized with isoflurane and FITC-inulin was injected into tail vein in the dose 40 mg/kg BW, tip of tail was cut to collect capillary blood within two hours. One tail snip was done to collect ten plasma samples and FITC-inulin concentration was measured with NanoDrop-3300 fluoro-spectrometer (ThermoFisher Waltham, MA 02451). Obtained data were used in two-phase exponential decay fitting in GraphPad Prizm to calculate glomerular filtration rate.

Data analysis, justification of sample size and number of animals

Data analysis, justification of sample size and determining the number of animals to use in the protocols were approached as previously described in Chapter 2.

Results

Control-fed group (received 30 grams of regular 0.4% Na chow daily)

Our laboratory has measured the food intake for the ALMS1 KO and WT rats. Based on previous data measuring food intake for these two groups, we know that the ALMS1 KO rats ingest approximately 35 g of chow daily while the WT (Dahl) SS rats ingest approximately 27 g of chow daily. For our control-fed group, we wanted to provide enough food (close to normal conditions) to allow the ALMS1 KO rats to increase their body weight higher than that of the WT (Dahl) SS rats; while avoiding the dramatic increase in body weight for the ALMS1 KO group. For our protocol we used younger male rats approximately 6 weeks of age to determine if a larger body weight influences the blood pressure in the ALMS1 KO rats. However, we chose to give 30 g of regular 0.4% chow daily in consideration of the higher daily food intake by the WT (Dahl) SS rats. When

looking at the results for body weight in Figure 33, our data show that the ALMS1 KO rats had a larger BW compared to the WT (Dahl SS) rats at 11 weeks of age (KO: 358±8 vs. WT: 306±17g, $p<0.05$), 12 weeks of age (KO: 386±11 vs. WT: 328±16 g, $p<0.05$) and 19 weeks of age (KO: 448±14 vs. WT: 370±17g, $p<0.05$).

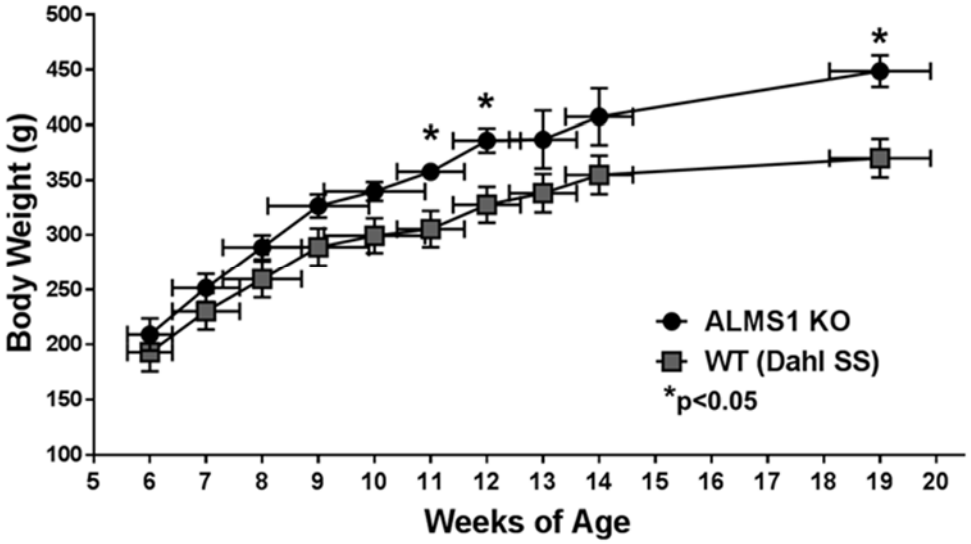


Figure 33. Body weight for the ALMS1 KO and WT (Dahl) SS rats for the control-fed group. Control fed ALMS1 KO rats receiving 30 grams of regular (0.4% Na) chow had a larger BW compared to the WT (Dahl SS) rats receiving the same dietary intake. Both groups consist of 6 male rats in which the ages of the rats at the time BW was obtained is noted along the x-axis of the graph. The ALMS1 KO rats had a larger BW compared to the WT (Dahl SS) rats at 11weeks of age (KO: 358±8 vs. WT: 306±17 g, $p<0.05$), 12 weeks of age (KO: 386±11 vs. WT: 328±16 g, $p<0.05$) and 19 weeks of age (KO: 448±14 vs. WT: 370±17 g, $p<0.05$).

More so, we were able to show once again (Figure 34) that the KO rats have an overall higher systolic blood pressure (SBP) compared to the WT rats. At 7 weeks of age (KO: 156±5 vs. WT: 142±3 mmHg, $p<0.05$), 10 weeks of age (KO: 160±3 vs. WT: 145±2 mmHg, $p<0.05$), 11 weeks of age (KO: 163±2 vs. WT: 147±5 mmHg, $p<0.05$), 13 weeks of age (KO: 174±7 vs. WT: 155±4 mmHg, $p<0.05$ and 15 weeks of age (KO: 180±3 vs. WT: 162±5 mmHg, $p<0.05$).

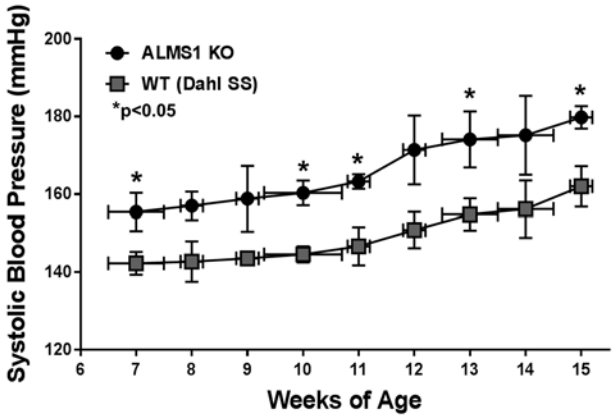


Figure 34. Systolic blood pressure for the ALMS1 KO and WT (Dahl) SS rats for the control-fed group. Control fed ALMS1 KO rats receiving 30 grams of regular (0.4% Na) chow had a higher blood pressure compared to the WT (Dahl SS) rats receiving the same dietary intake. Both groups consist of 6 male rats in which the ages of the rats at the time BW was obtained is noted along the x-axis of the graph. The ALMS1 KO rats had a higher BP compared to the WT (Dahl SS) rats at 7 weeks of age (KO: 156±5 vs. WT: 142±3 mmHg,*$p<0.05$), 10 weeks of age (KO: 160±3 vs. WT: 145±2 mmHg, $p<0.05$), 11 weeks of age (KO: 163±2 vs. WT: 147±5 mmHg, $p<0.05$), 13 weeks of age (KO: 174±7 vs. WT: 155±4 mmHg, $p<0.05$ and 15 weeks of age (*KO: 180±3 vs. WT: 162±5 mmHg, $p<0.05$).

Overall, what stands out is that the younger ALMS1 KO rats between the ages of 6 to 10 weeks old are hypertensive despite an equivalent body weight to the WT (Dahl) SS rats. These data suggest that the younger ALMS1 KO rat is hypertensive before they obtain a larger body weight. However, the cholesterol and triglyceride levels should also be measured before concluding about these results.

Moreover, we performed glucose tolerance test (GTT) in which the results in Figure 35 show that despite the difference in body weight and SBP, there was no difference between the groups for the fasting glucose level response until 14 weeks of age. More so, the fasting baseline blood glucose levels were higher in the KO rats at 10 weeks of age (KO: 83.7 ± 1.9 vs. WT: 71 ± 2.9 mg/dL, $p<0.05$) and 14 weeks of age (KO: 101 ± 5.3 vs. WT: 65.3 ± 3.5 mg/dL, $p<0.05$). These data suggest that the ALMS1 KO rats develop a decrease in glucose tolerance with growth. And also, these data suggest that the ALMS1 KO have higher higher circulating baseline blood glucose levels which may be a consequence of impaired glucose uptake and this is further explored in Chaper 4.

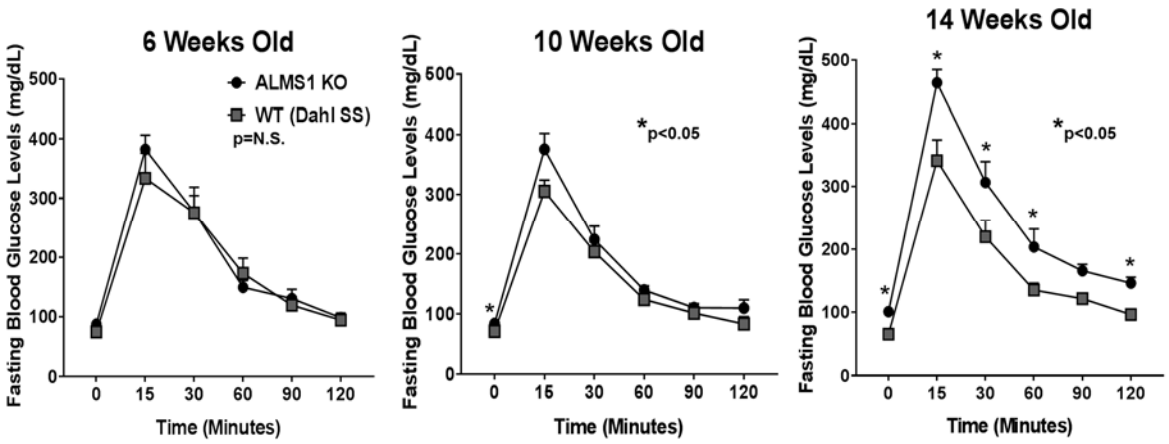


Figure 35. Fasting blood glucose tolerance test were performed approximately every 4 weeks in the both groups of rats while receiving 30 grams of regular chow daily. Separation between the two groups for the fasting glucose response was noted at 14 weeks of age at which time the ALMS1 KO rats had a greater glucose level response compared to the WT (Dahl SS) rats (*p<0.05, *KO vs. WT). Also, the control fed ALMS1 KO rats had a greater baseline fasting glucose level at 10 (KO: 83.7±2 vs. WT: 71±3 mg/mL, p<0.05) and 14 (KO: 101±5 vs. WT: 65±4 mg/mL, p<0.025) weeks of age compared to the WT (Dahl SS) rats.

Since the ALMS1 KO rats have higher fasting blood glucose levels we then chose to measure insulin to determine if there was a difference between the two groups. Figure 37 show that at 11 weeks of age the ALMS1 KO rats have a higher insulin compared to the WT (Dahl) SS rats (KO: 3.4±0.3 vs. WT: 1.3±0.4 ng/mL, p<0.05) and 15 weeks of age (KO: 4.2±1.3 vs. WT: 0.74±0.4 ng/mL, p<0.05) was greater in the ALMS1 KO rats. Furthermore, we obtained serum leptin measurements (Figure 37) to evaluate the cause

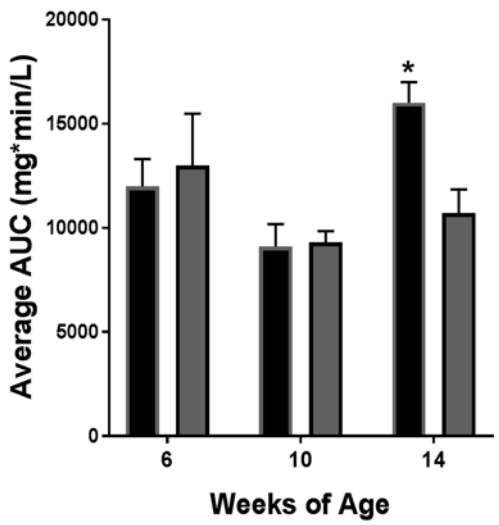


Figure 36. Control-fed group area under the curve for blood glucose tolerance tests. At 6 and 10 weeks of age there was no difference in glucose levels between the ALMS1 KO and WT (Dahl SS) rats. Separation between the two groups were noted at 14 weeks of age at which time the ALMS1 KO rats had a greater glucose level response compared to the WT (Dahl SS) rats (KO: 16010 ± 990 vs. WT: 10713 ± 1132 mg*min/L, *p<0.05) in which the AUC was also greater for the ALMS1 KO rats.

for hyperphagia in the ALMS1 KO rats. The serum leptin level at 11 weeks of age (KO: 30625±2774 vs. WT: 2006±355 pg/mL, p<0.025) and 15 weeks of age (KO: 39517±3844 vs. WT: 1954±171 pg/mL, p<0.025) was greater in the ALMS1 KO rats. Overall, these data suggest that the ALMS1 KO rats have hyperinsulinemia and hyperleptinemia. Possible explanations for these results are explored in Chapter 4.

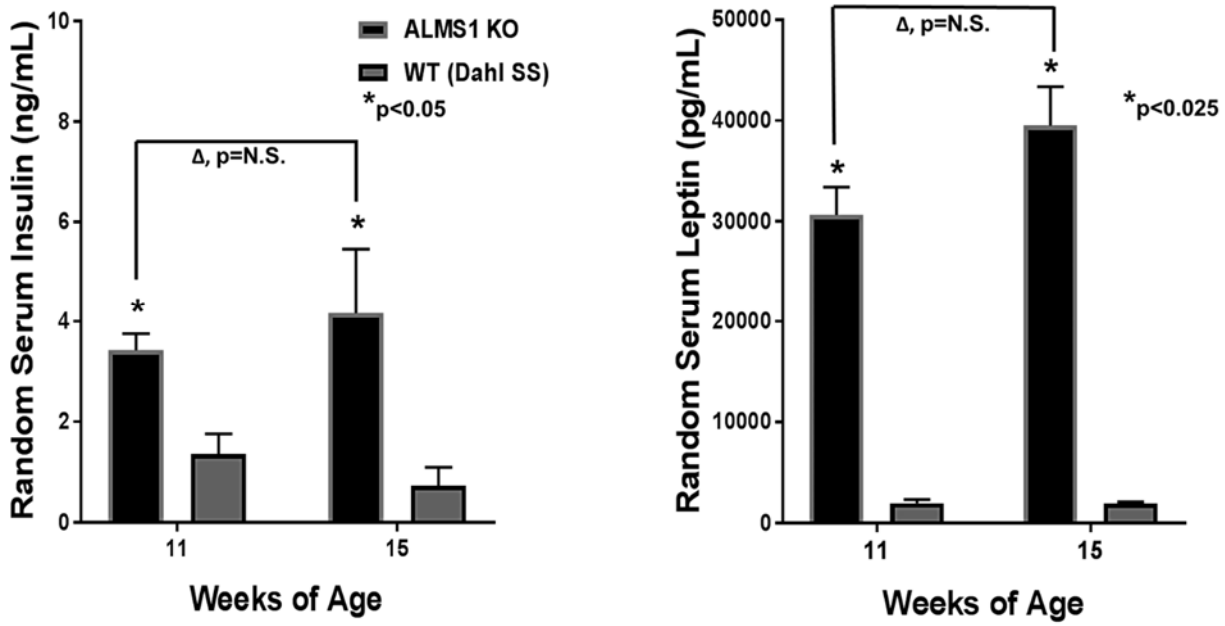


Figure 37. **Random serum insulin and random serum leptin levels in the ALMS1 KO and WT (Dahl) SS rats for the control-fed group.** The ALMS1 KO rats maintained a higher random serum insulin level and random leptin level throughout the experiment in comparison to the WT (Dahl SS) rats. The random serum insulin level at 11 weeks of age (KO: 3.4±0.3 vs. WT: 1.3±0.4 ng/mL, p<0.05) and 15 weeks of age (KO: 4.2±1.3 vs. WT: 0.74±0.4 ng/mL, p<0.05) was greater in the ALMS1 KO rats. The random serum leptin level at 11 weeks of age (KO: 30625±2774 vs. WT: 2006±355 pg/mL, p<0.025) and 15 weeks of age (KO: 39517±3844 vs. WT: 1954±171 pg/mL, p<0.025) was greater in the ALMS1 KO rats.

Lastly, we wanted to determine the renal Na urine excretion in response to diuretics in the ALMS1 KO rats. Before proceeding with this experiment we measured the filtering capacity of the kidneys by obtaining the glomerular filtration rate (GFR) via FITC-inulin measurements (Figure 38) and discovered at 20 weeks old the KO rats have a lower GFR compared to the WT rats (KO: 0.57±0.1 vs. *WT:1.58±0.3 ml/min/gKW, p<0.05). This is opposite to what we previously found in younger (average of age 10

weeks old) ALMS1 KO rats with normal GFRs [69]. Therefore, these results we did not explore the influence of the NKCC2 cotransporter in blood pressure (BP) elevation for the KO rats. Previous literature has shown that diuretics are not as effective with a lower GFR as a result of decrease luminal availability and when applicable are required at higher doses in order to lower blood pressure [4, 116]. However, in regards to the ALMS1 KO rats, the decline in their renal filtering capacity as they grow will continue to pose an issue for comparing urinary Na excretion between both groups of rats.

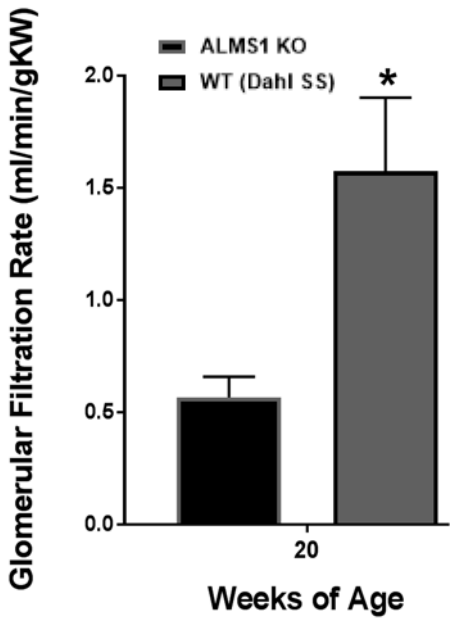


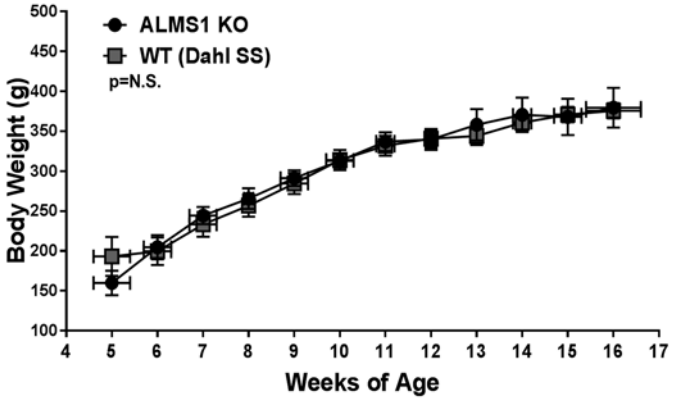
Figure 38. Glomerular filtration rate (GFR) determined using the fixed inulin measurement for the ALMS1 KO and WT (Dahl) SS rats in the control-fed group. We used fluorescein isothiocyanate (FITC)-inulin GFR measurements and found the ALMS1 KO rats have a lower glomerular filtration rate compared to the WT rats at 20 weeks of age (KO: 0.57±0.1 vs. *WT: 1.58±0.3, *p<0.05).

Caloric restricted (CR) group (receiving 24 grams of modified regular 0.4% Na chow daily)

For the caloric restricted group we chose a 20% caloric restriction in which the rats would receive 24 g daily of a modified (Envigo) version of the regular 0.4% chow daily. We chose 24 g of chow daily to ensure that all rats ate 100% of the food and as previously noted the WT (Dahl) SS rats consume approx. 27 g of chow daily. Also, we believe this caloric restriction would prevent the increase in body weight previously shown in the control-fed ALMS1 KO group.

When previewing the data for the caloric restricted (CR) group it is apparent that the decrease in caloric intake influences the BW without altering the elevation in BP for the KO rats. As depicted in Figure 39, for the 20% caloric restricted group, there was no difference in the body weight which suggest that we successfully prevented the separation in body weight in which the ALMS1 KO rats did not achieve a larger body weight. The body weight from 5-16 weeks of age for both groups were equivalent (KO: 302.5 ± 20.5 vs. WT: 300.3 ± 18.9 g, p=N.S.

Figure 39. Body weight for the ALMS1 KO and WT (Dahl) SS rats in the 20% caloric restricted group. The caloric restricted rats (fed 20% less calories than the control fed group) in which the ALMS1 KO and WT (Dahl SS) rats received 24 grams of a modified regular (0.4% Na) chow had an equivalent BW that was maintained throughout the experiment. Both groups consist of 7 male rats in which the ages of the rats are noted along the x-axis of the graph.



However, despite an equivalent body weight for the two groups, Figure 40 shows that the ALMS1 KO rats still have a higher SBP compared to the WT (Dahl) SS rats. The ALMS1 KO rats had a higher BP compared to the WT (Dahl SS) rats at 6 weeks of age (KO: 148±5 vs. WT: 134±3 mmHg, $p<0.05$), 10 weeks of age (KO: 162±6 vs. WT: 142±5 mmHg, $p<0.05$), 15 weeks of age (KO: 176±6 vs. WT: 156±5 mmHg, $p<0.05$), and 16 weeks of age (KO: 178±6 vs. WT: 156±2 mmHg, $p<0.05$).

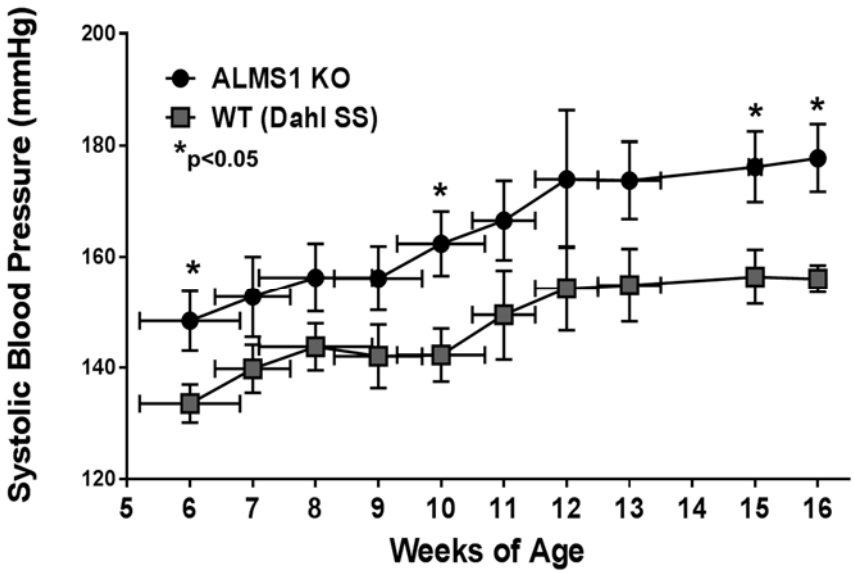


Figure 40. Systolic blood pressure for the ALMS1 KO and WT (Dahl) SS rats in the 20% caloric restricted group. The caloric restricted rats (fed 20% less calories than the control fed group) chow had a higher blood pressure compared to the WT (Dahl SS) rats receiving the same dietary intake. Both groups consist of 7 male rats in which the ages of the rats at the time BW was obtained is noted along the x-axis of the graph. The ALMS1 KO rats had a higher BP compared to the WT (Dahl SS) rats at 6 weeks of age (KO: 148±5 vs. WT: 134±3 mmHg, $p<0.05$), 10 weeks of age (KO: 162±6 vs. WT: 142±5 mmHg, $p<0.05$), 15 weeks of age (KO: 176±6 vs. WT: 156±5 mmHg, $p<0.05$), and 16 weeks of age (KO: 178±6 vs. WT: 156±2 mmHg, $p<0.05$).

Interestingly, when looking at the results for the random GTT shown in Figure 41 there was a higher blood glucose level for the ALMS1 KO compared to the WT (Dahl SS) rats at all ages.

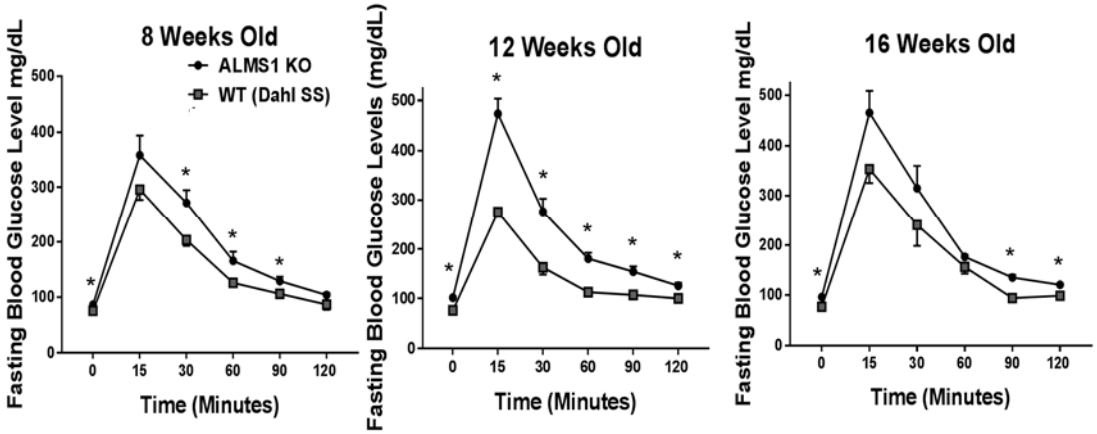


Figure 41. Fasting blood glucose tolerance test were performed approximately every 4 weeks in the both groups of rats while receiving 24 grams of the modified regular chow daily. At all ages (8, 12 and 16 weeks old) the ALMS1 KO rats had an overall higher blood glucose response compared to the WT rats. * $p<0.05$

Figure 42 shows the area under the curve (AUC) for each GTT. The AUC was greater for the ALMS1 KO rats at 8 weeks of age (KO: 12175 ± 1318 vs. WT: 8845 ± 703.4 mg*min/L, $p<0.05$), 12 weeks of age (KO: 13822± 1622 vs. WT: 7394 ± 607.7 mg*min/L, $p<0.05$) and 16 weeks of age (KO: 16115 ± 1466 vs. WT: 11349 ± 1239 mg*min/L, $p<0.05$).

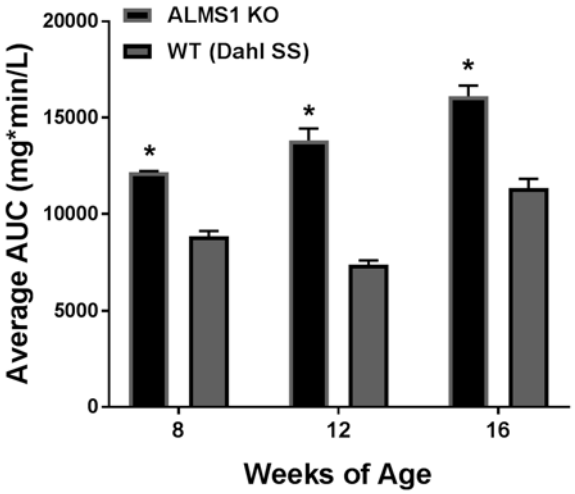


Figure 42. 20% Caloric restricted group area under the curve for blood glucose tolerance tests. 20% Caloric restricted group area under the curve for the glucose tolerance tests. In response to the 20% caloric restriction, the area under the curve (AUC) was higher for the ALMS1 KO group at all ages compared to the WT (Dahl) SS. There were 7 male rats per group. At 8 weeks old the KO: 12175 ± 1318 vs. WT: 8845 ± 703.4, $p<0.05$. At 12 weeks old the KO: 13822 ± 1622 vs. WT: 7394 ± 607.7, $p<0.05$. At 16 weeks old, the KO: 16115 ± 1466

Moreover, the 20% caloric restricted ALMS1 KO rats had a greater baseline fasting blood glucose level at 8 (KO: 86±3 vs. WT: 75±3 mg/dL, $p<0.05$), 12 (KO: 102±5 vs. WT: 76±2 mg/dL, $p<0.05$) and 16 (KO: 96±2 vs. WT: 77±3 mg/dL, $p<0.05$) weeks of age compared to the WT (Dahl SS) rats. These data suggest a progression of glucose intolerance in the ALMS1 KO with 20% caloric restriction. A decrease in glucose uptake as a result of impaired GLUT4 trafficking has been proposed as a possible explanation [37]. This is further explored in chapter 4.

Then we wanted to determine if caloric restriction would have an effect on the serum insulin and serum leptin levels in the ALMS1 KO rats. Figure 43 shows that the caloric restricted ALMS1 KO rats maintained a higher serum insulin level and random leptin level throughout the experiment in comparison to the WT (Dahl SS) rats.

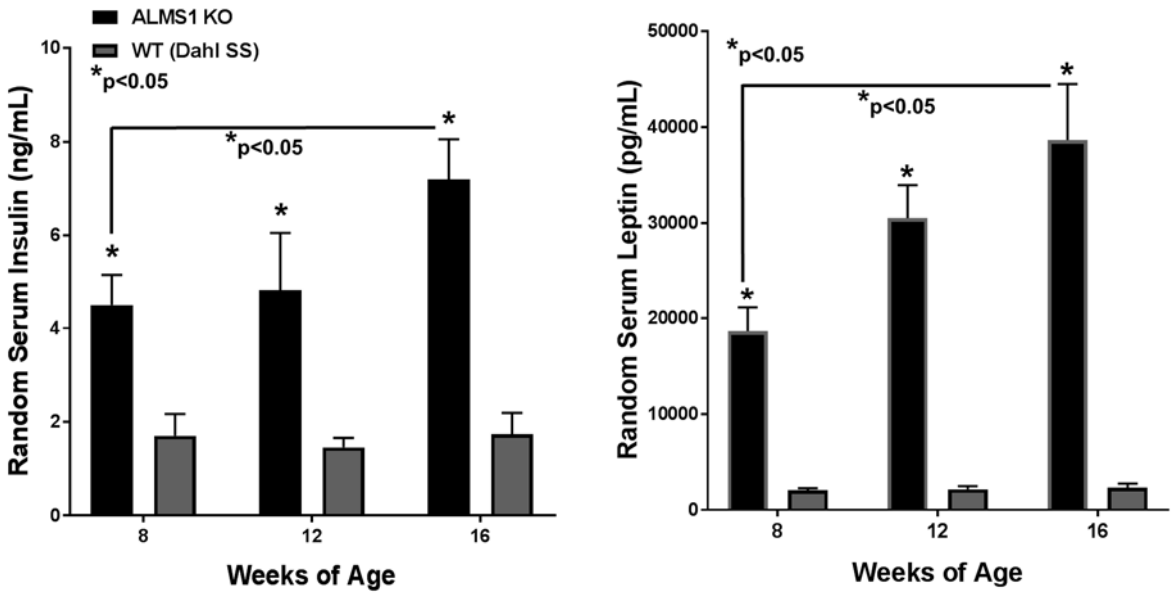


Figure 43. Random serum insulin and random serum leptin levels in the ALMS1 KO and WT (Dahl) SS rats for the caloric restricted group. The ALMS1 KO rats maintained a higher random serum insulin level and random leptin level throughout the experiment in comparison to the WT (Dahl SS) rats. The random serum insulin level at 8 weeks of age (KO: 4.5± 0.7 vs. WT: 1.7 ±0.5 ng/mL, p<0.05), 12 weeks of age (KO: 4.8±1.2 vs. WT: 1.5±0.2 ng/mL, p<0.05) and 16 weeks of age (KO: 7.2±0.9 vs. WT: 1.7±0.5 ng/mL, p<0.05) was greater in the ALMS1 KO rats. The random serum leptin level at 8 weeks of age (KO: 18664±2457 vs. WT: 2023±246 pg/mL, p<0.025), 12 weeks of age (KO: 30537±3432 vs. WT: 2159±308 pg/mL, p<0.025) and 16 weeks of age (KO: 38673±5834 vs. WT: 2296±467 pg/mL, p<0.025) was also greater in the ALMS1 KO rats.

The serum insulin level at 8 weeks of age (KO: 4.5± 0.7 vs. WT: 1.7 ±0.5 ng/mL, p<0.05), 12 weeks of age (KO: 4.8±1.2 vs. WT: 1.5±0.2 ng/mL, p<0.05) and 16 weeks of age (KO: 7.2±0.9 vs. WT: 1.7±0.5 ng/mL, p<0.05) was greater in the ALMS1 KO rats. The serum leptin level at 8 weeks of age (KO: 18664±2457 vs. WT: 2023±246 pg/mL, p<0.001), 12 weeks of age (KO: 30537±3432 vs. WT: 2159±308 pg/mL, p<0.001) and 16 weeks of age (KO: 38673±5834 vs. WT: 2296±467 pg/mL, p<0.001) was also greater in the ALMS1 KO rats. These data suggest that hyperinsulinemia and hyperleptinemia still exist in the ALMS1 KO rats despite a 20% caloric restriction.

Summary

Overall, these data strongly suggest that the genetic deletion of ALMS1 increases

blood pressure (BP) elevation independently of body weight. Our data indicate that the larger body weight in the KO rats is not the underlying cause for a higher BP. More so, our data demonstrates that with caloric restriction there is an improvement in BW but this does not appear to diminish the development of glucose intolerance. Despite the reduced calories the ALMS1 KO: caloric restricted group had a higher AUC at 11 weeks of age compared to the ALMS1 KO: control-fed group. One plausible explanation for the progression of glucose intolerance in the ALMS1 KO rats with caloric restriction is an impaired insulin-stimulated GLUT4 translocation as suggested by Favaretto, et al. [38]. However, the opposite is seen at 11 weeks for the WT (Dahl) SS: caloric restricted group which had a lower AUC compared to the WT (Dahl) SS: control-fed group. This suggest that the WT (Dahl) SS rats appear to have a lowering of the area under the curve with less caloric intake.

Also, the random leptin levels did not appear to be influenced by the caloric restriction. The leptin was still higher in the KO rats regardless of diet and age. The leptin resistance displayed by the KO rats (both control-fed and CR) could be the result of changes in the leptin receptor sensitivity which compromises proper signaling from the brain to the body to stop eating (satiety) [9, 49]. This has been studied in other rat models but in order to make this conclusion in regards to the ALMS1 KO rat further experiments are necessary. Lastly, the GFR for the control-fed group of ALMS1 KO rats was lower in comparison to the WT: control-fed rats. This suggests that the KO rats have decreased renal filtering capacity that progresses with age. This may also reflect a progression of renal disease secondary to ALMS1 deletion [9, 33, 47]. However, more studies (such as creatinine clearance) are necessary to provide further insight for to investigate GFR in this rat model.

CHAPTER 4: SUMMARY OF RESULTS AND DISCUSSION

Summary of results

The underlying mechanisms involved in the onset of hypertension and more so, salt-sensitive hypertension still remains unclear. In humans diagnosed with Alström Syndrome (ALMS), hypertension and chronic kidney disease are just some of several co-morbidities that occur in this patient population [84]. Previously, our lab showed that proteins interacting with the NKCC2 have an effect on the activity of this cotransporter [6, 8, 22]. Also, our lab discovered that the ALMS1 protein interacts with the carboxyl (C2)-NKCC2 [69]. In collaboration with the rat genome editing consortium at the Medical college of Wisconsin (MCW) we generated the ALMS1 KO rats in a (dahl) salt sensitive background, to investigate the underlying cause of blood pressure elevation in these rats. In doing so we considered the involvement of nephron segments such as the proximal tubule, thick ascending limb (TAL), distal convoluted tubule, and collecting duct. In order to explore ALMS1 role in blood pressure regulation, we used only younger (4-9 week old) male rats for our experiments, to avoid confounding effects of obesity.

First, we showed that the ALMS1 KO rats have a higher SBP compared to the WT (Dahl) SS rats which is present regardless of the dietary Na intake (normal salt: 0.22% and high salt: 4% Na chow). Our data for blood pressure measurements was consistent regardless of our approach (noninvasive tail cuff vs. invasive radio-telemetry). And our blood pressure data suggesting hypertension in the ALMS1 KO rats is supported by previously published data in Alström Syndrome patients [86, 88-90]. Overall, our data confirm that both the ALMS1 KO and WT (Dahl) SS rats are salt sensitive which is shown with the increase in blood pressure with a higher dietary Na intake (e.g. increasing the dietary Na intake from .22% (normal) to 4% (high) Na chow). Yet, the ALMS1 KO rats have a greater

increase in blood pressure with the 4% Na chow which suggests a higher state of Na retention and/or salt sensitivity. Moreover, we are the first to explore salt sensitivity in response to ALMS1 deletion. Plus, we obtained blood pressure measurements in the rats while giving oral bumetanide (3mg/kg daily) to inhibit NKCC2 and found that both the ALMS1 KO and WT (Dahl) SS groups had an equivalent decrease in blood pressure which suggests that there is enhanced NKCC2 activity in both groups. However, unlike the WT (Dahl) SS rats, the ALMS1 KO rats did not have restoration of blood pressure (i.e. a return of blood pressure to baseline blood pressure measurements) while receiving a high (4% Na) diet with bumetanide. These results suggest that other mechanisms may be involved in the elevation of blood pressure in the ALMS1 KO rat model. So we then chose to further evaluate NKCC2 involvement while determining the involvement of other nephron segments.

Next, we used the metabolic cage protocol to study the effect of several diuretics on renal Na excretion in the ALMS1 KO rats. This included the loop diuretic, bumetanide (NKCC2 inhibitor), acetazolamide (carbonic anhydrase inhibitor), hydrochlorothiazide (HCTZ, NCC inhibitor), as well as benzamil and amiloride (ENaC inhibitors). Our results showed that bumetanide caused a higher natriuretic response in the ALMS1 KO rats which suggest that in the absence of this inhibition there is increased NaCl reabsorption along the TAL via enhanced activity of the NKCC2 in the ALMS1 KO rats. On the other hand, acetazolamide, HCTZ, benzamil and amiloride resulted in a lower natriuretic response in the ALMS1 KO rats; and the results from these experiments showed that there was higher natriuresis in the WT (Dahl) SS rats. Therefore, these data supports our hypothesis that blood pressure elevation in the ALMS1 KO rat is related to enhance NKCC2 activity along the thick ascending limb (TAL). However, as previously noted from the data obtained with

the telemetry protocol, other mechanisms may be involved in the elevation of blood pressure for the ALMS1 KO rats.

Based on preliminary data that was collected by me and my former colleague (Dr. Jaykumar), we measured body weight and food intake for the ALMS1 KO and WT (Dahl) SS rats. We found that ALMS1 KO rats are hyperphagic (abnormal food appetite involving excessive eating) and have a larger body weight in comparison to the WT (Dahl) SS rats starting at 10-11 weeks of age. Obesity has been well characterized in the human population with Alström Syndrome, as well as the ALMS1 mutant mouse model [9, 38, 68, 87-89]. So we then chose to explore if obesity influences the elevation of blood pressure in the ALMS1 KO rats. This decision was based on several factors starting with the telemetry data shown in chapter 2; where we did not have restoration of BP with bumetanide alone which suggest that other mechanisms are involved in the hypertension for the ALMS1 KO rats. More so, when exploring whether other nephron segments were involved in renal Na handling for the KO rats, our data suggest that enhanced renal NaCl reabsorption is NKCC2-indepenent.

We designed a protocol that allowed us to prevent the increase in body weight for the ALMS1 KO rats and perform different experiments to determine if the hypertension in this rat model was dependent on obesity and insulin resistance. We had a total of four (4) groups: Control-fed: ALMS1 KO and Control-fed: WT (Dahl) SS rats (n=6 per group) vs. Caloric restricted: ALMS1 KO and Caloric restricted: WT (Dahl) SS rats (n=7 per group) that were not all done simultaneously because of availability and number of experiments. The control-fed group received 30g of regular 0.4% Na chow daily while the caloric restricted group received 24g of a modified version of the regular 0.4% Na chow (created with Envigo) which allowed both groups to receive 0.12g of Na daily regardless of the

caloric intake. We found that the 20% caloric restriction successfully prevented the increase in body weight for the ALMS1 KO rats; and when looking at the control-fed vs. caloric groups it appears proper growth was maintained for the caloric restricted groups. However, other parameters such as tibia bone length would need to be obtained before concluding the caloric restricted rats did not have growth restrictions. Moreover, we found that the ALMS1 KO rats are hypertensive with normalization of body weight but with hyperinsulinemia, as well as hyperleptinemia in the control-fed and caloric restricted ALMS1 KO groups. Then we discovered that caloric restriction decreased the body weight, but according to the GGT test results, did not modify glucose tolerance in the ALMS1 KO rats; yet, it seemed to increase glucose tolerance in the WT (Dahl) SS rats. Overall, our data suggest that the ALMS1 KO rats are hypertensive independent of obesity; and it is plausible that insulin resistance developed independently of body weight.

Discussion

Our ALMS1 KO rat model is extremely complex and there are other laboratories that have shown that deletion of ALMS1 in cells can lead to impaired cilia formation, and therefore decrease or effect the intracellular trafficking of enzymes, hormones, etc. which ultimately affect organ function [32-33, 38, 78, 82 , 114]. The ALMS1 gene is expressed in different organs such as the brain, eyes, heart, pancreas, liver, kidneys and reproductive organs [56, 60, 69]. Our lab is interested in determining how ALMS1 deletion would affect blood pressure and renal salt handling in the rat model.

In this section we discuss the results from our protocols while exploring the possible explanations for the data obtained in our rat model. First, as previously mentioned we are the first to explore Na retention in response to ALMS1 deletion. When comparing the two groups of rats, our results suggest that the deletion of ALMS1 enhances salt sensitivity and

this increase in Na retention is related in part to enhance NKCC2 activity in the TAL.

Our laboratory previously found that proteins interacting with NKCC2 could change the activity of this cotransporter, thereby increasing NaCl absorption in the thick ascending limb (TAL) which can cause an elevation of blood pressure (BP) [6-8, 69]. Using a proteomics-approach, Drs. Caceres and Jaykumar, identified ALMS1 as a potential interacting partner of the carboxyl (C2)-NKCC2 [69]. Bumetanide and furosemide are both NKCC2 inhibitors that can be used in rat models; but bumetanide is a more potent loop diuretic such that 1mg of bumetanide is equivalent to 40mg of furosemide [119]. Given our goal was to achieve the maximum natriuretic effect we chose to use bumetanide 20mg/kg for the metabolic cage protocol [69-70]. Bumetanide induced a higher natriuretic response in the ALMS1 KO rats suggesting enhanced Na reabsorption via increased NKCC2 activity in the TAL. However, our telemetry data shows that both groups have an equivalent decrease in blood pressure with bumetanide which suggests that both the KO and WT rats have increased Na reabsorption in the TAL [6, 50, 69, 110]; yet, with maximum inhibition of NKCC2 cotransporter, we have shown that ALMS1 deletion causes a higher NaCl reabsorption along the TAL in dahl-salt sensitive rats.

Then we explored the proximal tubule by using the carbonic anhydrase (CA) inhibitor, acetazolamide (50mg/kg). In humans, acetazolamide is predominantly used to treat glaucoma, epilepsy and elevated intracranial pressure [29, 109]. We chose acetazolamide because it's known to influence the proximal tubule by inhibiting CA enzyme which ultimately allows reabsorption of bicarbonate, sodium, and chloride [29, 109]. By inhibiting CA, these ions are excreted, along with excess water in the urine which serves to lower the blood pressure. For our protocol, we found that acetazolamide induced a lower natriuresis in the ALMS1 KO rat. Further investigation is required in order to determine if

our results are an accurate reflection of proximal tubule sodium reabsorption in the ALMS1 KO rats. There could be other reasons (e.g. lower plasma Na level and/or altered proximal tubule Na reabsorption secondary to distal nephron Na handling) why the NaCl absorption in the proximal tubule is not higher in ALMS1 KO rats.

Also, we evaluated the Na-Cl cotransporter (NCC) located in the distal convoluted tubule [46]. When giving the NCC inhibitor, hydrochlorothiazide (HCTZ) at a dose of 200mg/kg, the ALMS1 KO rats had a lower natriuretic response compared to the WT (Dahl) SS rats. A possible explanation for these results is that the deletion of ALMS1 could result in lower function of the NCC cotransporter to compensate for higher NKCC2 activity. There is a medical condition called Gitelman syndrome, is an autosomal recessive kidney disorder caused by genetic mutations resulting in improper function of the NCC; characterized by low blood levels of potassium and magnesium, decreased excretion of calcium in the urine, elevated blood pH and a low blood pressure [94]. So if the deletion ALMS1 caused a decrease in NCC activity, we would expect a decrease in blood pressure. Thus, it is unlikely that ALMS1 regulates NCC directly.

Finally, there is the epithelial Na channel (ENaC) located in the collecting duct. When exploring the ENaC inhibitors (benzamil 10mg/kg and amiloride 20mg/kg) we found that the results showed a lower natriuresis in the ALMS1 KO rat. The ENaC is located in a very critical position for the regulation (or homeostasis) of the extracellular fluid volume, electrolyte balance and long term BP [116]. For example, under normal conditions factors such as high-salt intake decreases ENaC activity and the ENaC expression level at the apical membrane surface of the cortical collecting duct and this demonstrates the critical role of ENaC in enhanced Na reabsorption and water retention [116]. And there are researchers that support ENaC playing an important role in BP regulation, not only via the

kidney, but also in other tissues commonly involved in BP regulation [21, 116, 120]. More so, ENaC in the central nervous system is proposed to regulate BP via sympathetic nervous system activity and recent evidence suggests that ENaC contributes to vascular function and the myogenic response [21, 116, 118, 120]. Yet, our results showed a lower natriutresis in response to both ENaC inhibitors (benzamil and amiloride) which suggested that renal ENaC is not playing a critical role in blood pressure elevation of the ALMS1 KO rats.

Ultimately, more research in the ALMS1 KO rats is necessary in order to determine the causes for a lower natriuretic response to the diuretics targeting the proximal tubule, distal convoluted tubule and collecting duct. Nevertheless, there are still other possible mechanisms that could increase blood pressure elevation in the ALMS1 KO rats.

For instance, we found that our ALMS1 KO rats have an increase in body over time that suggest progressive obesity with growth. Obesity is a common phenotypical characteristic for the human population diagnosed with Alström syndrome, and also has been used to describe the ALMS1 mutant mouse and fat aussie mouse models [9, 38, 86-88]. It has been suggested that obesity increases the risk of hypertension secondary to activation of the sympathetic nervous system (SNS) [72]. A case study done by Ni-Chung Lee et al., showed that a reduction in calories in an 18 months old child with Alström syndrome reduced the body weight and delayed the age of onset for hyperinsulinemia and hypertriglyceridemia along with cardiac impairment in response to the caloric restriction [95]. Even though, this clinical study had 1 subject, the rationale for conducting this research validated the importance for determining if obesity was related to blood pressure elevation in the ALMS1 KO rats. And given that dahl-salt sensitive rats have been shown to develop metabolic syndrome over time we wanted to determine how metabolic parameters would be influenced by the genetic deletion of ALMS1 [42, 49, 50, 110].

We performed a protocol involving four groups of younger (4-7 week old) male rats for the control-fed: ALMS1 KO vs. WT (Dahl) SS and 20% caloric restricted: ALMS1 KO vs. WT (Dahl) SS which consisted of several experiments. The 20% caloric restriction was done to prevent the increase in body weight for the ALMS1 KO rats. We also wanted to evaluate insulin resistance in response to the reduced calories for this rat model. Our results suggest that the ALMS1 KO rats are hypertensive regardless of body weight. In the absence of ALMS1 deletion, it has been shown that obesity increases blood pressure through multiple mechanisms, including increased activity of the RAAS and renal sodium retention [72]. However, Dr. Jaykumar measured plasma renin and found it was higher in ALMS1 KO rats; yet, we did not explore SNA involvement in our present study [69].

We also found that the ALMS1 KO rats are hyperleptinemic regardless of caloric intake. Also, it is important to note that ALMS1 is expressed in the brain, more specifically the hypothalamus [54]. Leptin is an adipocyte-derived peptide (meaning it is synthesized and released by fat cells) and it functions to suppress appetite and increase thermogenesis (production of body heat) while raising SNS activity and blood pressure [72, 111]. Our results show that the ALMS1 KO rats are hyperleptinemic starting at a younger age (approximately 8 weeks old) and these results appear to be consistent with other obese animal models [9, 38, 72 ,111]. This suggests that there is leptin insensitivity in the ALMS1 KO rats due to leptin receptor dysfunction and/or impaired hypothalamic signaling cascade. A paper titled, "A Truncating Mutation of Alms1 Reduces the Number of Hypothalamic Neuronal Cilia in Obese Mice" showed that mice with ALMS1 mutations have a decrease in adenylyl cyclase 3 (AC3) function which has been associated with increased body weight and fat mass [54]. However, leptin receptor function and hypothalamic signaling have not been studied in this rat model; and additional testing would be necessary before determining the cause of

hyperleptinemia and whether it is related to the hypertension seen in the ALMS1 KO rats.

Furthermore, our data demonstrates that the ALMS1 KO rats have hyperinsulinemia regardless of caloric intake in which glucose intolerance still exists in the younger ALMS1 KO rats with caloric restriction. We hypothesize that this may be secondary to altered insulin-stimulated GLUT4 trafficking to the plasma membrane as implied by Favaretto et al. [38]. This notion is further supported by the observed higher area under the curve values obtained in the caloric restricted ALMS1 KO group. Yet, GLUT4 trafficking must be evaluated in the ALMS1 KO rats before concluding that this is the cause of the glucose tolerance seen in this rat model.

Finally, our data revealed that the KO rats have a decline in GFR at 18-20 weeks of age, which may correlate with an increase in age [19, 35, 39]. We previously found that younger rats approximately 10 weeks of age have a normal GFR [67]. More so, the ALMS1 KO rats have both a larger body weight, and also a larger kidney weight. However, evaluating the GFR at different age intervals is necessary before concluding that aging is associated with a reduction in GFR for the KO rats. Therefore, the data suggest that the ALMS1 KO rats have a lower renal filtering capacity in comparison to the WT (Dahl) SS rats. Recently, some genome-wide-association studies (GWAS) have linked eighty (80) ALMS1 mutations in the general population with decreased renal function and renal disease [40, 80, 102, 127]. So our data suggesting a decline in renal function for the ALMS1 KO rats is supported by a characterized decline in renal function and renal disease found in the human population diagnosed with Alström syndrome [9, 38, 40, 82-85, 87]. Ultimately, more testing is needed in order to conclude that the possibilities proposed in this discussion are mechanisms involved in the phenotypic characteristics seen in the ALMS1 KO rat model [124].

The ALMS1 KO rat is a complex model, and thus far the phenotypic characteristics seen in our rat and other mice models correspond to those of the human population diagnosed with Alström Syndrome (ALMS) [9, 38, 82-85, 87]. The onset of hypertension (HTN) and salt sensitivity in the ALMS1 KO rat may be driven by enhanced renal Na absorption along the thick ascending limb via the NKCC2 cotransporter. However, it is possible that other underlying mechanisms are involved. Furthermore, there are single nucleotide polymorphisms (SNPs) in ALMS1 that have been associated with obesity and decreased renal function in the general population [10, 19, 69, 103, 106]. So studying the association of the ALMS1 gene with hypertension in the general population is of most importance. Our research could increase awareness within the medical and research communities and help improve the medication regimen, medical decision making, and ultimately the medical outcome for patients with Alström syndrome, renal impairment/disease, type 2 diabetes, cardiac disease, hypertension and resistant hypertension, etc. A continuation of this research could better delineate a cascade of events that would better enhance our understanding of the underlying mechanisms responsible for the onset of hypertension and enhanced salt sensitivity in the ALMS1 KO rat model.

Strengths and limitations of the study

Strengths:

We utilized the advantage of successfully generating this global knockout rat of ALMS1 for testing our hypothesis.

1. Rats have been used for many years to replicate human diseases. More specifically, using rats as animal subjects is ideal for kidney-related research because more is known about their renal physiology, and also how the rat anatomy resembles the anatomy of human beings.

2. The ALMS1 KO rats were obtained from the MCW and were generated in a Dahl salt sensitive (SS) background. Since Dahl SS rats are known to be salt sensitive, we determined whether the genetic deletion of ALMS1 in these rats would increase blood pressure and exacerbate salt sensitivity. [69]
3. The ALMS1 rat colony was managed by our lab and this was one of my responsibilities as a pre-doctoral student in the lab. This allowed rats to be selected based on genotype (KO vs. WT), litter group and date of birth. Therefore, by strategically selecting each rat for the KO vs. WT groups we decreased the variability in our study.
4. In all our protocols, the KO and WT rats were littermates and this decreased the variability in our results. Also, there is reduced genetic variability in the human population as well.
5. Our data could be used as a means for collaborating with medical researchers to facilitate a broader understanding of renal function and metabolic alterations in the Alström syndrome patient population.

Limitations:

Despite well conserved basic principles of cellular and renal physiology between rodents and humans, some limitations do exist. However, the kind of research we conducted here would have been difficult to perform in human subjects.

Following are some specific limitations related to our study:

1. Zinc finger nuclease gene editing technology has been known to have some issues such as off-target effects [69]. However, the metabolic characteristics seen in the ALMS1 KO rats have been previously reported in different ALMS1 mutant mice models indicating that these effects are likely due to ALMS1 deletion and not due

to the influence of off-target effects. We tested this hypothesis and thus far our data is consistent with what has been published in the ALMS1 mutant mouse model [9, 38, 54]. However, more experiments (using controls) are necessary to determine if there are other unknowns for the rat model.

2. Rats were grouped based on genotype (KO vs. WT) and we strived to be consistent with the age range for both groups since our studies used age as a variable when evaluating the results for SBP, BW, GTT, etc. At times this proved problematic because it resulted in an age differences within the group. Yet it is difficult to avoid this issue if the effect size group number, n, is to be obtained at this time of initiating the experiment. This is a consequence of the ALMS1 colony rat breeders being heterozygous, and therefore of the pups born, 25% were ALMS1 KO while another 25% of pups were WT (Dahl) SS.
3. Obtaining BP measurements with noninvasive tail-cuff instead of invasive radio-telemetry has some disadvantages. The data obtained using tail cuff is from the selected day(s) of running the experiment and usually represents an average of 1 hour of BP measurements; whereas, with radio-telemetry more data can accumulate from 24-hours of measurements, and does not require the presence of an operator. More so, data analysis with radio-telemetry is simple and less error is present given less stress from little to no handling of the animals. However, as stated earlier we strived to reduce the variability of our data by having one researcher (myself) conduct all the experiments and based on data analysis for the BP measurements both tail cuff and radio-telemetry, resulted in similar results overall.
4. Some of our experiments lacked time controls that would be used to validate the

changes occurring in various parameters (e.g. blood pressure, body weight, blood glucose response, etc.) to be a result of ALMS1 deletion and not some other underlying variable.

5. Measuring transport directly (e.g. isolated perfused tubules) in order to determine the involvement of other nephron segments is preferred and would be important for validating the metabolic cage data for the several diuretics that were used here in the ALMS1 KO rats. However, this is a protocol our laboratory will consider performing in the near future.
6. The ALMS1 KO rats have a reduced GFR that can result in a lower diuretic-induced natriuresis which could be an issue in the older rats [4, 114-116]. This made it difficult to compare the natriuretic effect of different diuretics between groups (KO vs. WT). Calculating the fractional excretion of sodium for both groups of rats could have provided further insight about the renal sodium handling; however, the serum sodium levels would be needed for both groups.
7. In the telemetry protocol (Figure 5) a single-dose of bumetanide was used to lower blood pressure. Using various doses of bumetanide, to create a dose-response curve would have been most preferred for investigating NKCC2 involvement in the ALMS1 KO rat model. However, this would had taken several months to perform because different groups of KO and WT rats (preferably at younger ages) would had been required to obtain reliable results.

Perspectives

Currently, the mechanism and genes responsible for salt-sensitive hypertension in humans and animal models are unclear. Only a few genes have been identified to be directly involved in salt sensitivity [7, 8, 131]. The role of ALMS1 in hypertension, renal

function and renal salt absorption had only been studied by our laboratory [69]. Our lab used a newly generated ALMS1 knockout rat model, to study salt sensitivity and the effect of obesity in renal function.

The National Health and Nutrition Examination Surveys (NHANES) 2011-2012 found that 29.1% of adults aged 18 years and older have hypertension [77]. It has been shown that approximately half of the hypertensive population is sensitive to salt intake [23, 96]. The kidney has been implicated in the development of many hypertension; in particular salt-sensitive hypertension [16, 23, 32, 40, 79]. More specifically, the nephron segment called the thick ascending limb (TAL) is in part responsible for enhanced salt retention in animal models of salt sensitive hypertension [5-8, 79]. Our research is studying a novel mechanism and a specific gene that may be involved in the development of the hypertension, and more so in salt-sensitive hypertension in the general population. We have shown here that deletion of the ALMS1 protein, enhances NKCC2-mediated reabsorption of NaCl by the thick ascending limb, which contributed to hypertension during high salt intake. This is the first data to show that ALMS1 is involved in salt sensitivity and ALMS1 involvement in a specific nephron segment. Moreover, we have demonstrated that the elevation of blood pressure in response to ALMS1 deletion is independent from body weight. Yet, we do not know if hypertension in the ALMS1 KO rat is independent from insulin resistance and leptin resistance.

In humans, ALMS1 mutations cause renal disease and metabolic alterations along with other physiological abnormalities that ultimately lead to co-morbidities which are both detrimental and costly to our society [69, 86, 88-90]. Enhancing our understanding about ALMS1 and how the deletion of this gene influences blood pressure and renal NaCl transport can improve the outcome for both Alström syndrome patients and the general

population. It would be very important to further our research using human subjects (ideally in larger cohorts) who have ALMS1 mutations or single-nucleotide polymorphisms (SNPs). This is enable us to elucidate the effects of the ALMS1 gene on blood pressure regulation.

APPENDIX

IACUC Protocol Approval Letters

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 02-12-15

Protocol Effective Period: March 1, 2015 – February 28, 2018

TO: Keyona King-Medina
Physiology
5374 Scott Hall

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 02-12-15

"The Role of Alms1 in the Development of Salt-Sensitive Hypertension (ADMIN)"

DATE: February 27, 2015

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **March 1, 2015** through **February 28, 2018**. The listed source of funding for the protocol is **NIH**. Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active.

The work on the project will not involve the use of live animals conducted within Wayne State University or John D. Dingell VAMC facilities. The principal investigator conducting this work requires an approved IACUC protocol for the live animal work conducted in that facility. However, be aware that the grantee always retains the primary responsibility for ensuring compliance with PHS Policy.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Research Administration
One Ford Place, 2F
Detroit, MI 48202
(313) 874-4360 Office

April 17, 2017

To: Pablo Ortiz

Fm: Pamela Harding, Ph.D., Chair
Carol Vich, Vice Chair
Institutional Animal Care and Use Committee

Re: **"The role of ALMS1 and PREX1 in Renal Function and Blood Pressure"**
(IACUC No. 1547)

Period of IACUC Approval: **April 17, 2017 through April 16, 2020**

Number of Animals Approved: 2182 rats and 546 mice

SD rats	1143	**C57 Bl6J mice**	250
Alms1 KO rats	306	**Alms1 KO mice**	204
PREX1 KO rats	214	**Alms1 loxp flanked**	23
Dahl Salt-Sensitive rats	519	**Renal-specific ALMS1 KO**	46
		Renal specific Cre	23

Dear Dr. Ortiz:

Thank you for submitting an application for review by the Institutional Animal Care and Use Committee. The application was presented at a convened meeting on February 03, 2017. A revised application responding to IACUC concerns was reviewed and all concerns were adequately addressed.

The expiration date for this study is April 16, 2020. In order to remain compliant with federal regulations, a new protocol application must be submitted to the IACUC office prior to expiration if you intend to proceed with this line of work beyond the above indicated expiration date. A Final Report must be submitted at the completion of the 3-year approval period or upon early termination of the project.

Any adverse effects on animals must be reported to the IACUC as soon as possible.

A copy of the signed and stamped protocol indicating approval of the Institutional Animal Care and Use Committee is enclosed for your files. Please feel free to contact The IACUC Coordinator at 874-4360 if you have any questions regarding this matter.

Enc.

cc: Bioresources

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ABSTRACT

THE ROLE OF ALSTRÖM SYNDROME 1 (ALMS1) IN HYPERTENSION AND SALT SENSITIVITY AND METABOLIC SYNDROME

by

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In humans, inactivating mutations in the Alström syndrome 1 (ALMS1) gene cause obesity, insulin resistance and hypertension (metabolic syndrome). More so, SNPs in the ALMS1 gene have been associated with kidney disease and cardiovascular disease. The mechanisms causing these alterations are unclear. We recently found that the ALMS1 is expressed in the kidney thick ascending limb (TAL) where it mediates endocytosis of the renal Na/K/2Cl cotransporter termed NKCC2. To study the role of ALMS1 in renal physiology we generated ALMS1 knockout (KO) rats in a Dahl salt-sensitive genetic background via zinc-finger nuclease targeting. We previously found that the amount of NKCC2 in the apical surface is higher in the TALs from ALMS1 KO rats compared to WT (Dahl) SS (WT) rats. We hypothesized that deletion of ALMS1 will increase SBP and enhance salt-sensitivity of BP, in part due to higher NKCC2-mediated Na reabsorption. We used noninvasive tail cuff measurements and invasive radio-telemetry to study the effects of dietary sodium (normal salt: .22% and high salt: 4% Na chow) on the systolic blood pressure (SBP) of the KO rats. The tail cuff measurements revealed with 0.22% Na chow the ALMS1 KO rats had a higher SBP than the WT rats (KO: 136±3 and WT: 125±3 mmHg, $p<0.05$). Then radio-telemetry confirmed the KO rats have a higher baseline SBP

on .22% Na chow (KO: 145±2 and WT: 134±1 mmHg, p<0.025). After 2 weeks on high Na intake (4% Na chow) the SBP in ALMS1 KO rats increased to 181±1 mmHg, a 35±3 mmHg increase, whereas the SBP increased to 159±2 mmHg in WT rats, a 25±1 mmHg increase (p<0.025 vs ALMS1 KO). Then upon giving a daily dose of bumetanide (3mg/kg), an NKCC2 inhibitor, the SBP decreased in both groups (KO: -23±4 and WT: -30±6 mmHg, p=0.40). After 6 days of treatment with bumetanide, the SBP was normalized only in the WT rats while the SBP remained elevated in the ALMS1 KO rats (KO: 131±3 and WT: 115±4 mmHg, p<0.025). We hypothesize that ALMS1 deletion leads to hypertension due to a decrease ability to excrete a salt load secondary to enhanced NaCl transport by the TAL and other nephron segments. Using conscious rats in metabolic cages, it took longer for KO rats to excrete the Na load overall in which at all time points (6, 9, 24 hour) the urinary Na excretion was lower in KO rats (cumulative Na, KO: 4251±590 vs. WT: 8788±994 μmols/24h, p<0.025). To study the role of different nephron segments in Na reabsorption we used a maximal single dose of segment-specific diuretic and measured urine Na and volume excretion. The TAL diuretic bumetanide 20mg/kg, induced a higher natriuretic response in KO rats (KO: 485.2±37.1 vs. WT: 221.8±32 μmols/8h, p<0.05). Contrarily, acetazolamide, HCTZ and benzamil resulted in a lower natriuretic response in the KO rats; suggesting in absence of the inhibition with these diuretics there is no increase Na reabsorption along the proximal tubule, distal convoluted tubule and collecting duct. Our data showed that KO have a decreased ability to excrete a salt load, and also the deletion of ALMS1 increases the sensitivity to TAL diuretics but not to acetazolamide, HCTZ or benzamil which is in part due to enhanced Na transport by the TAL. Furthermore, we found that deletion of ALMS1 in rats causes progressive obesity and insulin resistance evidenced by the growth charts and glucose tolerance tests

obtained in our previous protocols. So it is unclear whether increases in blood pressure occur prior or after the development of metabolic alterations in the KO. So we studied the effect caloric restriction (CR) on body weight (BW), SBP, glucose tolerance and leptin levels. A control-fed group of KO and WT rats received 30 g/day regular chow (0.4% Na). A caloric restricted (CR) group received 20% less food (24 g/day of a modified regular chow (0.4%). We hypothesized that hypertension in the KO rats is primarily caused by a renal defect and not secondary to the metabolic syndrome. For the control-fed group we used young KO and WT rats (5-7 weeks old) with 6 male rats per group. At 5-7 weeks of age KO rats had similar body weights (KO: 239±16.18 g vs. WT: 230±16.64 g, N.S.); the baseline glucose was similar with a similar AUC. However, SBP was already higher in KO rats (KO: 156±5 vs. WT: 143±3 mmHg, $p<0.025$). Four weeks later the body weight began to diverge between strains (ALMS1 KO: 326±10 g vs. WT: 282±17 g, $p<0.05$). Baseline glucose was higher in KO (KO: 84±2 vs. WT: 71±3 mg/dL, $p<0.005$) and glucose tolerance test produced a larger AUC (KO: 11915±1349 vs. WT: 9176±9155 mg*min/dL). The SBP continued to be elevated in the KO rats (KO: 160±3 vs. WT: 144±3 mmHg, $p<0.05$). The 20% caloric restricted group had 7 male rats per group. At 7 weeks of age, the rats had a similar BW (KO: 209±13 g vs. WT: 182±15 g), but the baseline glucose was higher (KO: 86±3 vs. WT: 75± mg/dL, $p<0.05$), as well as the GTT. More importantly, SBP was higher in ALMS1 KO rats (KO: 156±5 vs. WT: 143±3 mmHg, $p<0.05$). Caloric restriction for 4 weeks prevented the increase in BW (KO: 359±8 vs. WT: 340±3 g, N.S.) but did not influence SBP or glucose intolerance. Therefore we conclude that the hypertension in the KO rats occur independently of obesity and insulin resistance. Furthermore, the caloric restriction prevents the increase in BW but does not prevent hypertension or improve metabolic alterations in this rat model. We conclude that the

ALMS1 KO rats are hypertensive and salt sensitive in part due to enhanced Na reabsorption along the TAL via the NKCC2 cotransporter which is independent of obesity and insulin resistance. To our knowledge this is the first rat model of metabolic syndrome with established hypertension that does not require a high salt diet. Ongoing experiments with this rat model will explore the other physiological systems involvement in blood pressure regulation/elevation in the ALMS1 KO rats.

AUTOBIOGRAPHICAL STATEMENT

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Education

Wayne State University, Detroit, MI	B.S.	05/2004	Biology
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2013	Women of Wayne Incentive Scholarship Program, Wayne State University
2013	Wayne State University Graduate and Physiology Dept. Dissertation Research Scholarship Recipient
2013	The President's Commission on the Status of Women (COSW) Scholarship 2013, Wayne State University
2007-2018	Physician Assistant's Task Force: Physician Assistant License, Lansing, MI.
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Contributions to Science

1. **Keyona N King-Medina** and Pablo Ortiz. Hypertension Precedes Metabolic Syndrome in the ALMS1 (Alström Syndrome 1) Knockout Rat (submission pending), 2019.
2. **Keyona N King-Medina**, Ankita B Jaykumar and Pablo Ortiz. Hypertension and Salt Sensitivity in the ALMS1 Knockout Rat (submission pending), September 2018.
3. Emily Henson, **Keyona N. King-Medina**, Jose Garcia-Pedraza, Pablo Ortiz. A Fructose- but Not a Glucose-enriched Diet, Induces a Salt-dependent Increase in Blood Pressure in Normal Rats (submission pending), September 2018.
4. Shukla, V., **King, K.N**., Ram, J.L. Zebra mussels at the freshwater/sea interface: Ionic and osmotic challenges to oocyte structural integrity. Abstracts, Annual Meeting of the Society for Integrative and Comparative Biology http://sicb.org/meetings/2003/schedule/ (http://sicb.org/meetings/2003/schedule/), Jan., 2003 (Integr Comp. Biol. 42: 1312, 2002).
5. Ram JL, Shukla, F., and **King, K.N.** Zebra mussels at the freshwater/sea interface: Ionic and osmotic challenges to oocyte integrity. Inv. Reprod. Devt. 45: 83-89, 2004.

Research Supports

1. F31HL123285 King-Medina (PI) 04/01/2015-03/31/2018
   NIH/NHLBI The Role of ALMS1 in Renal Function and Hypertension.

   The overall goal of this project is to study genetic deletion of ALMS1 in rats to determine if it causes hypertension and enhances salt-sensitivity of blood pressure, in part by increasing renal NaCl reabsorption by stimulation of NKCC2 in the thick ascending limb; and to determine if hypertension and NKCC2 activation in ALMS1 Knockout rats is independent of obesity.