**UNIVERSITA' VITA-SALUTE SAN RAFFAELE**

**CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE**

**Curriculum in Cellular and Molecular Biology**

# *Lss* KNOCK-IN MOUSE MODEL OF SALT-SENSITIVE HYPERTENSION AND RENAL GLOMERULAR DAMAGE

DoS: Prof. Paolo Manunta

Second Supervisor: Prof. Davor Pavlovic

Tesi di DOTTORATO di RICERCA di Sipontina Faienza

Matr. 015789

Ciclo di dottorato XXXV

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# DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

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All the results presented here were obtained by myself, except for:

1) CRISPR-Cas9, Surveyor assay, ESCs manipulation and chimeric mice generation (Results, paragraph 3.1) were performed by Core Facility for Conditional Mutagenesis (CFCM) form San Raffaele Scientific Institute, Milan, Italy

2) Analysis on blood and urine samples (Results, paragraph 3.3 for the Table 1 and 3.5 for the Table 2 were performed by Prof. Alessia Giordano from Department di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Milan, Italy

3) RNASeq and relative analysis (Results, paragraph 3.7, Figure 15 A e B) were performed by LIFE & BRAIN GmbH Biomedical & Scientific Technology Platform, Bonn, Germany

4) Histological evaluation of slices and scoring (results, paragraph 3.6, Figure 14B) was performed by Dr. Francesca Sanvito, San Raffaele Scientific Institute, Milan, Italy.

All sources of information are acknowledged by means of reference.

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## ABSTRACT

The blood pressure (BP) response to different salt intakes, the so-called salt sensitivity, shows great variability among individuals and is more frequent in hypertensive patients compared to the general population. Elevated levels of the steroid hormone Endogenous Ouabain (EO) have been associated with hypertension (HT) and salt sensitivity.

We mainly characterized the missense variation rs2254524 (Val642Leu; V642: C variant; L642: A variant) of the Lanosterol Synthase (*LSS*), a gene coding for a key enzyme in steroid biosynthesis, since AA patients on a low salt diet showed a greater reduction in BP compared to the LSS CC and only CC patients had an increase in plasma EO after the low salt protocol. Hence, we hypothesized that LSS could affect salt-sensitive HT by regulating EO biosynthesis.

To dissect the functional correlation between *LSS* polymorphism, EO, and salt-sensitive hypertension, we generated by CRISPR-Cas9 a knock-in mouse model carrying the *Lss* V643L mutation, homologous to the human V642L variant, expressed ubiquitously.

Male mice were fed with a normal salt diet (0.5% NaCl), high salt diet (4% NaCl), or low salt diet (<0.03% Na), and BP was measured every two days by the BP-2000 blood pressure tail-cuff system, in conscious trained mice.

At baseline, the V643L mutation affected kidney weight normalized to body weight that was significantly enlarged at 3 and 12 months of age, compared to the WT counterpart.

The *Lss* V643L mutation did not affect EO biosynthesis and SBP at 3 and 12 months, *per se*, but affects SBP responsiveness to salt intake. Indeed, we observed an increased SBP upon a high-salt diet only in $Lss^{V643L/V643L}$ mice 12 months old compared to the control diet. Only the $Lss^{V643L/V643L}$ mice in a high-salt diet showed cardiac hypertrophy and a higher incidence of heart fibrosis compared to WT at 12 months. Moreover, the *Lss* mRNA level was differently regulated by a high-salt diet in the adrenal gland, liver, and heart of $Lss^{V643L/V643L}$ mice.

These mutant mice did not show any difference in kidney functionality and morphology.

In conclusion, the new *Lss* mouse model is similar to salt-sensitive hypertension phenotype observed in hypertensive patients because increases BP during high salt diet and develops cardiac damage and provides a good model of salt-sensitive hypertension. Moreover, our results reveal a role of the *Lss* gene in the regulation of BP upon salt intake variation.

# TABLE OF CONTENTS

## ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin-converting enzyme |
| ADD | Adducin |
| Ang II | Angiotensin II |
| ANP | Atrial natriuretic peptide |
| ASO-PCR | Allele-specific oligonucleotide-PCR |
| $AT_1$ | Angiotensin II type 1 receptor |
| AT1R | Angiotensin type 1 receptor |
| BP | Blood pressure |
| BUN | Blood Urea Nitrogen |
| CCR2 | CC chemokine receptor 2 |
| CKD | Chronic kidney disease |
| CLCNKA | Chloride channels CLC-Ka |
| CTS | Cardiotonic steroid |
| CVD | Cardiovascular disease |
| Cyp11b2 | Cytochrome P450 family 11 subfamily B member 2 |
| CYP3A3 | Cytochrome P450 family 3 subfamily A member 3 |
| CYP3A4 | Cytochrome P450 family 3 subfamily A member 4 |
| CYP3A5 | Cytochrome P450 family 3 subfamily A member 5 |
| CYP3A7 | cytochrome P450 family 3 subfamily A member 7 |
| DBP | Diastolic blood pressure |
| DC | Dendritic cell |
| ECV | Extracellular volume |
| EGFR | Epidermal growth factor receptor |
| ENaC | Epithelial sodium channel |
| EO | Endogenous ouabain |
| ESC | Embryonic stem cell |
| FGF23 | Fibroblast growth factor 23 |
| GWAS | Genome-wide association study |
| HDR | Homology directed repair |
| HSD | High salt diet |

| | |
|---|---|
| HTN | Hypertension |
| HyperPath | Hypertension Pathotype |
| IGF | Insulin-like growth factor |
| ISS | Inverse salt-sensitive |
| IL-1β | Interleukin 1β |
| IL-23 | Interleukin 23 |
| IL-6 | Interleukin 6 |
| KL | Klotho |
| KO | Knock-out |
| KI | Knock-in |
| LSD | Low salt diet |
| LSS | Lanosterol synthase |
| MBP | Mean blood pressure |
| MHR | Milan hypertensive rats |
| MR | Mineralocorticoid receptor |
| NAFLD | Nonalcoholic fatty liver disease |
| NCC | $Na^+$-$Cl^+$-cotransporter |
| NCKX3 | Sodium/calcium/potassium exchanger |
| NCX1 | Sodium/calcium exchanger |
| NEDD4L | NEDD4 like E3 ubiquitin-protein ligase |
| NHE3 | Sodium–hydrogen antiporter 3 |
| NKA | $Na^+$/$K^+$-ATPase |
| NKCC2 | Solute carrier family 12 member 2 |
| NO | Nitric oxide |
| NSD | Normal salt diet |
| PAM | Protospacer adjacent motif |
| Pllp | Plasmolipin |
| PRKG1 | Protein kinase cGMP-dependent 1 |
| RAAS | Renin-angiotensin-aldosterone system |
| RAG | Recombination-activating gene |
| RAS | Renin-angiotensin system |
| Ren | Renin |

| | |
|---|---|
| RFLP | Restriction Fragment Length Polymorphism |
| SBP | Systolic blood pressure |
| SLC24A3 | Solute carrier family 24 member 3 |
| SLC8A1 | Solute carrier family 8 member 1 |
| SNP | Single nucleotide polymorphism |
| SNS | Sympathetic nervous system |
| Src | Proto-oncogene tyrosine-protein kinase Src |
| SS | Salt sensitivity or salt-sensitive |
| SR | Salt-resistant |
| SVR | Systemic vascular resistance |
| TAL | Thin ascending limb of Henle's loop |
| UMOD | Uromodulin |
| WNK1 | WNK lysine deficient protein kinase 1 |

## LIST OF FIGURES AND TABLES

# 1. INTRODUCTION

## 1.1 Systemic arterial hypertension

Hypertension (HTN) is a dangerous condition characterized by a great increase in blood pressure (BP). The BP level is determined by the amount of blood pumped by the heart - the cardiac output - and the peripheral resistance of the artery wall to the blood flow – systemic vascular resistance (SVR). BP levels show a Gaussian distribution in the general population.

A recent classification defines normal, high BP, hypertension (stages 1 and 2), and hypertensive crisis by a score based on BP level. According to this classification, hypertension is diagnosed if the systolic blood pressure (SBP) overcomes the limit of 140 mmHg and/or the diastolic blood pressure (DBP) is higher than 90 mmHg (Williams *et al*, 2018; Unger *et al*, 2020).

Hypertension remains the most important risk factor for cardiovascular diseases (CVDs) and renal diseases (Kjeldsen, 2018). CVDs are the leading cause of death worldwide and accounted for 32% of global deaths in 2019 and 44% of all deaths in 2010 in Italy (https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds); https://www.epicentro.iss.it/cardiovascolare/). In 2010, 1.38 billion adults globally had hypertension with a high prevalence (75%) in low- and middle-income countries (Mills *et al*, 2016). In essential (primary) hypertension, which affects 90-95% of hypertensive subjects, there are no specific detectable and treatable causes. In the rest of 5-10%, high BP is the result of different diseases that could affect the kidney, adrenal gland, heart, and vessels. This type of hypertension is called secondary hypertension and the treatment depends on the primary disease.

Although the specific cause of primary hypertension remains unclear, it is known its association with genetics and environmental risk factors.

### *1.1.1 Risk factors for hypertension*

The most important risk factor for hypertension is aging due to its physiological, economical, and social changing impacting the hypertension onset. The prevalence of hypertension increases with age: 76.4% of men and 79.9% of women aged over 75 years have hypertension in the U.S. (Mozaffarian *et al*, 2016). Moreover, older age groups

(≤60 years) are the most impacted in high-income countries whereas middled age groups (40-59 years) in low- and middle-income countries. These differences could be explained by disparities in the ease of access to the health care system, antihypertensive drugs availability, but also differences in risk factors such as physical inactivity, smoking, and unhealthy diet (Mills *et al*, 2016).

Other critical risk factor for hypertension and CVD is the overweight and obesity. The NHANES study shows that the prevalence of hypertension increases with the increase in BMI (Wang & Wang, 2004). Moreover, it was demonstrated by several studies that weight lowering correlates with BP reduction (Moore *et al*, 2005; The Trials of Hypertension Prevention Collaborative Research Group, 1997; Fantin *et al*, 2019).

Besides these leading risk factors, different studies demonstrated other several factors affecting the increase of BP. One risk factor of note is sex, indeed more women are affected by hypertension than men over 75 years older, probably because their life expectancy is longer than men (Tasić *et al*, 2022).

Previous studies have highlighted that lifestyle-related factors may impact on the HTN status. Among those the most known risk factor is the high sodium intake with the diet. Several studies demonstrate that excess sodium intake (NaCl) with the diet is a trigger of hypertension (Adrogué & Madias, 2007). Other risk factors correlated with the modern era are alcohol, smoking but also social and economic levels (Nagao *et al*, 2021).

### *1.1.2 Genetics of hypertension*

The SBP and DBP levels are hereditable (Levy *et al*, 2000). So far Genome Wide Association Studies (GWAS) have identified around 1000 SNPs associated with BP or hypertension (Evangelou *et al*, 2018; Lip & Padmanabhan, 2020). Over the years, several genetic approaches were used to deepen the genetics of HTN, starting from the simpler candidate gene approach to more recent GWAS, thus increasing our knowledge of the complex genetic architecture of BP regulation by identifying several genes and their variants associated with HTN onset or BP levels. To date, more than 30 genes and 1000 single nucleotide polymorphisms (SNPs) were associated with hypertension (Newton-Cheh *et al*, 2009; Evangelou *et al*, 2018; Giri *et al*, 2019 Lip & Padmanabhan, 2020). There are evidence of rare monogenic forms of hypertension, mainly results of mutation in genes such as *UMOD, CYP11B1, HSD11B2,* and *SCNN1B*, that have contributed to

understanding kidney and adrenal gland role in BP regulation (salt retention and steroids overproduction). However, in the case of primary HTN, multiple genetic variants just affect this phenotype, each of which has only a small effect on BP regulation as a canonical polygenic trait (common disease, common variant hypothesis) (Manolio *et al*, 2009).

## 1.2 Salt-sensitive hypertension

Salt sensitivity (SS) of BP is a physiological trait characterized by the BP level changes parallel to dietary sodium assumption (Elijovich *et al*, 2016). However, this response to sodium variations shows great variability among individuals, with SS more common in hypertensives (30-50%) compared to the general population (25%) (Weinberger *et al*, 1986; Elijovich *et al*, 2016). Moreover, aging, obesity, and other diseases affecting the kidney such as diabetes, increase the SS (Kotchen *et al*, 2013).

Although it was demonstrated by several studies that salt sensitivity is itself a risk factor for CVD and mortality (Bigazzi *et al*, 1994; Morimoto *et al*, 1997; Weinberger *et al*, 2001), in most countries the average daily assumption of sodium in the diet is above (~2-4 g/day) the physiological needed suggesting by the World Health Organization (<2 g/day) (World Health Organization, 2012)

### *1.2.1 Clinical evaluation of salt sensitivity*

One of the major problems in the diagnosis of salt sensitivity is that it is not currently standardized, therefore it is difficult to classify patients according to salt-sensitivity. Currently, the two main accepted methods for the clinical identification of salt sensitivity are the dietary protocols and the Weinberger test (Elijovich *et al*, 2016; Weinberger *et al*, 1986).

The dietary protocol consists of 2 weeks of diet: the first week of a strictly low salt diet followed by a high salt diet, and the BP was measured as the difference between the low and the high salt protocol as shown in the figure below (Figure 1) (Elijovich *et al*, 2016).

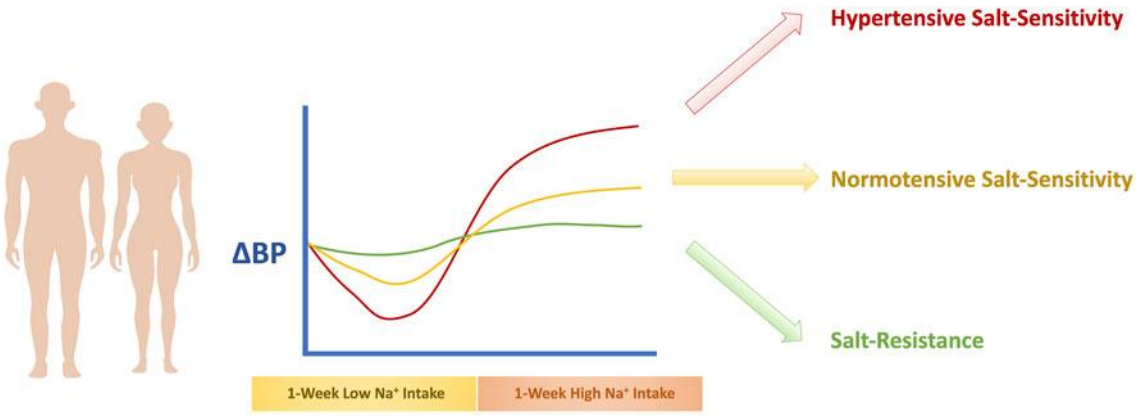


***Figure 1. BP salt sensitivity identification by diet***. *Schematic representation of one available method for salt sensitivity determination. Patients undergo 1 week of a low $Na^+$ diet followed by a second week of a high $Na^+$ diet. The BP variation (ΔBP) is calculated as the difference between the BP at the beginning and the end of the diet protocol. If the BP increases patients are classified as normotensive or hypertensive salt-sensitive, depending on the increasing amount. If the BP undergoes a minimal change, it is salt-resistant BP. Figure from Maaliki et al, 2022.*

The Weinberger test is a faster protocol for SS diagnosis. It consists of 2 liters infusion of saline solution over 4 h that causes an extracellular volume (ECV) expansion. The sodium and volume depletion were obtained by the administration of furosemide diuretic in patients under a low salt diet.

For the identification of salt sensitivity in our laboratory we used the acute sodium load test. This is a modified Weinberger test with an infusion of 2 liters of saline solution in 2 h, without the administration of loop diuretics and the BP was measured at the beginning and the end of the test (Citterio *et al*, 2020). As shown in Figure 2, three different responses of BP to acute sodium load test are possible: increasing, non-responding, and decreasing. These BP responses define the salt-sensitive (SS), salt-resistant (SR), and inverse salt-sensitive (ISS) patients' classification respectively (Cuka *et al*, 2022).

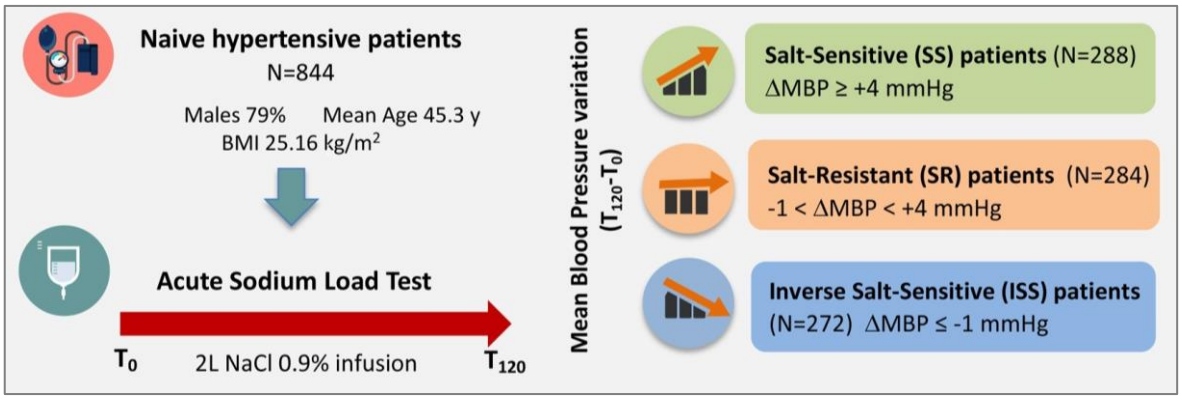


***Figure 2. Acute sodium load test and salt sensitivity.*** *Modified Weinberger test for salt sensitivity diagnosis consists of 2 liters of NaCl 0.9% infusion in 2 hours. The response of the mean blood pressure (MBP) to the sodium load, calculated as the difference between MBP at the beginning ($T_0$) and the end of infusion ($T_{120}$) identified SS, SR, and ISS patients. Adapted from Cuka et al. (2022).*

### *1.2.2 The kidney and the vaso-dysfunction theories in the onset of salt-sensitive hypertension*

The debate about the triggering event of BP increase after a NaCl load is still open, and two possible theories were postulated in which the kidney and vasculature are involved.

The prevailing theory for salt sensitivity determination – the volume-loading theory – argues that the trigger mechanism is the impairment of renal pressure natriuresis/diuresis relationship, that is the increase in renal sodium excretion because of mild increases in BP, typically due to extracellular fluid volume expansion, allowing BP to remain in the normal range. In salt-resistant in normal conditions, an increase in $Na^+$ intake causes a minimal change in BP due to ECV rise and maintenance. Moreover, the salt intake suppresses the renin-angiotensin-aldosterone system (RAAS), which inhibits sodium reabsorption (Guyton, 1992) (Figure 3).

In salt-sensitive individuals, where the kidney feedback regulation and/or humoral system and therefore the natriuresis is impaired, the expansion in ECV causes a persistent rise in BP. In the plot reported in Figure 3, the salt-sensitive subjects' slope is less steep than salt-resistant, meaning that the increase in salt intake causes a higher increase in the BP required to excrete excess water and salt (De Nicola *et al*, 2004) (Figure 3). In

hypertensives and patients affected by chronic kidney diseases (CKDs), the renal pressure natriuresis slope is shifted to the right of the plot (Figure 3).

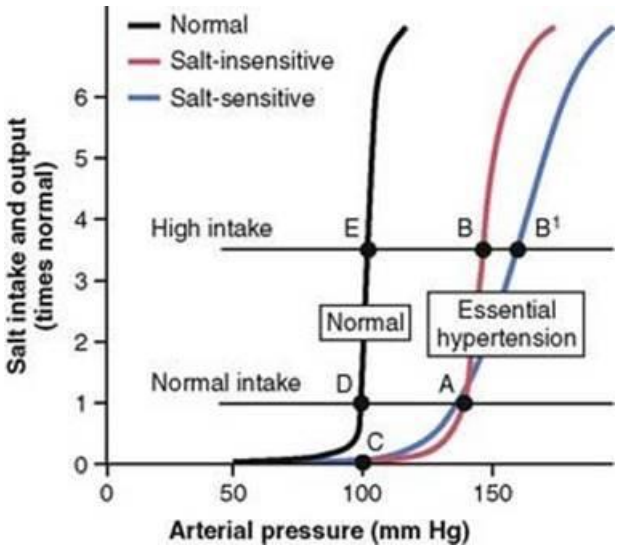


***Figure 3. Pressure natriuresis/diuresis relationship.*** *Plot representing the pressure natriuresis/diuresis slope of normal (black), salt-sensitive (blue), and salt-resistant (red) subjects. In essential hypertension, the curve was shifted to the right (B). The $B^1$ slope shows the BP increase necessary for salt excretion. Adapted from Guyton et al, (1980).*

In addition to the classical theory of the kidney's role in starting salt sensitivity, an alternative theory supports the impairment of vasculature constriction in the initiation of salt sensitivity.

The basis of this theory is that the $Na^+$ retention and cardiac output increase in salt-sensitive patients is quite like that of salt-resistant subjects. However, the decreasing of systemic vascular resistance to compensate for the cardiac output increase is a salt-resistant privilege, while salt-sensitive subjects fail in the systemic vascular resistance decreasing. This impairment of vascular resistance is responsible for the extracellular volume expansion and BP increasing in salt sensitivity (Figure 4) (Morris *et al*, 2016).

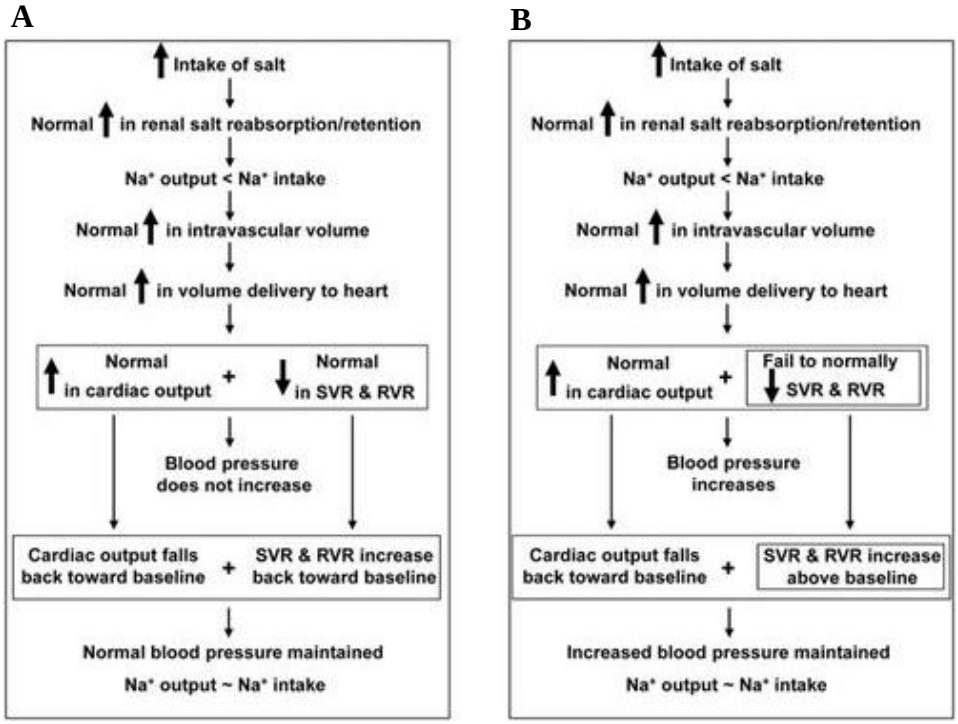


***Figure 4: The "Vasodysfunction" theory of NaCl-induced hypertension. A.*** *Mechanism of salt resistance. Usual pathway yielding a non-pressor response to NaCl loading in normal control subjects.* ***B.*** *Mechanism of salt sensitivity. Usual pathway yielding a pressor response to NaCl loading in affected subjects. The mechanism of salt sensitivity is responsible for the BP increase. Note that the failure in the response of systemic vascular resistance (SVR) is the hallmark of salt-sensitive induced hypertension. Adapted from Morris et al, (2016).*

### *1.2.3 Renin-angiotensin-aldosterone system modulation*

The long-term sodium balance and BP are tightly regulated by the renin-angiotensin-aldosterone system (RAAS), which directly affects vasoconstriction and diuresis. Even though the multiple components of this system, the two most important payers for sodium excretion and BP control are angiotensin II (Ang II) and aldosterone. Therefore, excessive activation of this system is a hallmark of salt-sensitive hypertension (Maaliki *et al*, 2022).

A drop in BP and fluid volume is sensed by juxtaglomerular cells in the kidney and causes the release of renin in the blood. The renin triggers the conversion of angiotensinogen to angiotensin I which in turn is cleaved to angiotensin II by the angiotensin-converting enzyme (ACE). Angiotensin II is the major effector of the system and exerts all functions through angiotensin II type 1 ($AT_1$) receptors. Ang II acts on different tissues and is

responsible for arteriolar vasoconstriction, water and sodium retention, and aldosterone release from the adrenal gland (Hall *et al*, 2012).
Increased but also low activity of the RAAS system increases the salt-sensitivity of the BP. Indeed, the salt-sensitive subjects showed an inadequate suppression of renin after high salt intake but also a reduced renin stimulation in response to salt depletion (Kobori *et al*, 2003; Yatabe *et al*, 2010).

Aldosterone is a mineralocorticoid hormone released from the adrenal gland in response to Ang II stimulation. Aldosterone acts on mineralocorticoid receptors (MR) which in turn act on tubular sodium and potassium reabsorption, regulating the BP (Ayuzawa & Fujita, 2021). Therefore, mutations that affect aldosterone production causes salt-sensitive hypertension. However, it was also demonstrated that salt-sensitive individuals have elevated levels of MR even if the aldosterone is inhibited, determined by Rac1 regulation. Indeed, Rac1 is a Rho family GTPase that directly activates MR in an aldosterone-independent manner, and it is activated by salt (Mishra *et al*, 2018).

Serum and glucocorticoid-regulated kinase 1 (SGK1) is an intracellular sensor of salt placed downstream of the MR pathway. SGK1 is upregulated by salt and activates the $Na^+$-$Cl^-$cotransporter (NCC) and epithelial sodium channel (ENaC) in the kidney (Farjah *et al*, 2003a; Maaliki *et al*, 2022). A rat model of salt-sensitive hypertension – Dahl rats- shows elevated levels of Rac1, SGK1 and EnaC compared to salt-resistant Sprague-Dawley rats (Farjah *et al*, 2003b; Aoi *et al*, 2007).

### *1.2.4 Other systems impaired in SS HTN*

Besides the kidney, vascular system, and mineralocorticoids, dysfunctions in other systems were identified as regulators of salt-sensitive hypertension.
Several studies in humans and mice have demonstrated that salt-sensitive subjects had an overactivation of the sympathetic nervous system (SNS) (Ando & Fujita, 2012). Moreover, the importance of SNS in the development of hypertension is confirmed by the fact that renal denervation lower BP and salt-sensitive hypertension (Symplicity HTN-2 Investigators, 2010). Fujita *et al*, (1980) demonstrated that salt-sensitive patients showed an increased level of norepinephrine compared to salt-resistant subjects. SNS overactivation could affect three different pathways: 1) increase renin secretion in

juxtaglomerular cells by $\beta_1$ – adrenoceptors, 2) decrease renal blood flow via $\alpha_{1A}$ – adrenoceptors, and 3) increase renal sodium reabsorption by increasing NCC activity via $\beta_2$AR-GR-WNK4 (di Bona, 2005; Fujita, 2014).

Several studies demonstrated that innate and adaptive immune systems have a role in arterial hypertension and salt-sensitive hypertension (Wenzel *et al*, 2016). Experiments on salt-sensitive rats treated with immunosuppressant ameliorated hypertension and decreased immune cell infiltration (Rodríguez-Iturbe *et al*, 2001). Moreover, RAG-1$^{-/-}$ mice, lacking T and B cells, showed a blunted BP increase due to Ang II infusion (Guzik *et al*, 2007). Kirabo *et al*, (2014) demonstrated that angiotensin II-infusion activates dendritic cells (DCs) with the consequent release of IL-6 and IL-1β and IL-23 that promote T cell activation and hypertension.

### *1.2.5 Genetic of salt-sensitive hypertension*

The interindividual differences observed in BP response to salt dietary changes could be also explained by genetics. On one hand, GWAS studies shed the light on numerous genes and their associated variants that can affect hypertension predisposition in the population (Newton-Cheh *et al*, 2009; Evangelou *et al*, 2018; Giri *et al*, 2019 Lip & Padmanabhan, 2020). On the other hand, the candidate gene approach identified genes and variants that are associated with salt-sensitive hypertension involved in both sodium reabsorption and vascular reactivity. These genes are summarized in Figure 5.

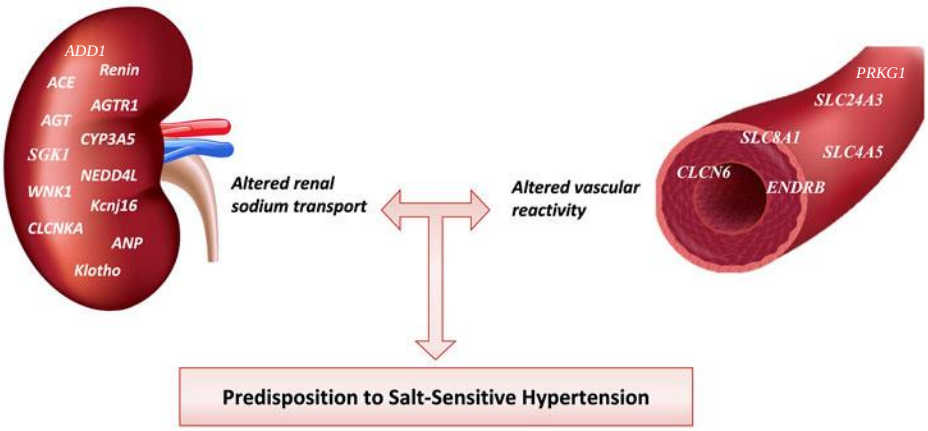


***Figure 5. Schematic representation of genes involved in the predisposition to salt-sensitive hypertension***. *The candidate gene approach identified different SNPs affecting genes that play key roles in kidney sodium handling and vascular reactivity. Figure modified from Maaliki et al, (2022).*

#### *1.2.5.1 Genes of the RAAS system*

The RAAS system plays a crucial role in sodium homeostasis and reasonably, different mutations and genetic variants identified in genes involved in the system are associated with salt-sensitive hypertension (Sanada *et al*, 2011).
The first step of RAAS activation is the release of renin from the juxtaglomerular cells in the kidney. $Ren^{-/-}$ knockout rats under a low salt diet show a reduction of 50 mmHg in SBP, in addition to abnormal kidney morphology and elevated blood urea nitrogen (BUN) and plasma creatinine level compared to the heterozygous counterpart (Moreno *et al*, 2011). $Ren^{-/-}$ rats have a reduced synthesis of aldosterone due to a reduction in *Cyp11b2* mRNA and protein levels (Raff *et al*, 2015). Moreover, it was demonstrated that renin deficiency is correlated with ion transport dysregulation mediated by sodium-hydrogen antiporter 3 (NHE3) and NCC (Pavlov *et al*, 2016).

Some polymorphisms in the gene upstream in the renin-angiotensin (RAS) chain was found in *ACE*. The insertion/deletion (I/D) polymorphism was evaluated in salt-sensitive patients. Patients homozygous for the insertion (II) displayed higher increased BP after a high-salt diet and a higher prevalence of hypertension compared to patients homozygous for the deletion (D/D) (Giner *et al*, 2000).
Angiotensin II exerts its action by angiotensin type 1 receptor ($AT_1R$) and promotes hypertension mainly through this activity. The KO of this receptor in the kidney is not sufficient for hypertension and cardiac hypertrophy onset in mice (Crowley *et al*, 2006).

Aldosterone and mineralocorticoid play important roles in salt-sensitive hypertension development. However, a variant in aldosterone synthase *CYP11B2* (rs179998) was not associated with salt sensitivity (Brand *et al*, 1999).

SGK1 is a kinase downstream aldosterone activity that regulates ENaC activity in the distal nephron. A study on the Hypertension Pathotype (HyperPath) group under a high salt diet identified 2 SNPs (rs2758151 and rs9402571) associated with high BP (Rao *et al*, 2013).

Cytochrome P450 (CYP) 3A group is composed of 4 isozymes: *CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7*. These genes encode monooxygenases which catalyze many

reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. CYP3A5 is expressed in the kidney and was associated with BP in patients (Ho *et al*, 2005; Fromm *et al*, 2005). *CYP3A5*1* allele (increased expression of enzyme) carriers have high BP compared to subjects carrying *CYP3A5*3* (reduced expression of enzyme) in a low salt diet (Eap *et al*, 2007).

#### *1.2.5.2* Renal transport-associated genes

There are no available studies about common variants in ENaC mutations associated with salt-sensitive hypertension. However, common variants in *WNK1* (rs880054 and rs2301880) and *NEDD4L* (rs4149601), two regulators of ENaC activation, were associated with increased hypertension and salt-sensitivity alone but especially when associated with variant in α-adducin (*ADD1*) G460W (Manunta *et al*, 2008a).

Adducin (ADD) is a membrane cytoskeleton protein that consists of ADD1, ADD2, and ADD3 subunits. ADD1 and ADD3 form heterodimers also present in the kidney and heart. ADD pay a critical role in actin-spectrin interaction and inhibit actin polymerization (Kiang & Leung, 2018). ADD1 was identified as a gene associated with hypertension in Milan hypertensive rats (MHR) (Bianchi *et al*, 1974). A point mutation (F316Y) identified in *Add1* is responsible for salt-sensitive hypertension observed in MHR rats. This mutation stimulates the Na-K pump internalization stimulating $Na^+$ reabsorption that in turn is responsible for hypertension (Bianchi, 2005). ADD1 SNP (rs4961) was also identified as associated with hypertension and salt-sensitive hypertension in humans (Casari *et al*, 1995; Cusi *et al*, 1997).

The chloride channels CLC-Ka is predominantly expressed in the kidney and mediates chloride transport in the thin ascending limb of Henle's loop (TAL). Loss of function mutations in the corresponding gene *CLCNKA* were associated with a deficit in NaCl reabsorption (Matsumura *et al*, 1999). rs848307, rs1739843, rs1010069, and rs1805152 variants of *CLCNKA* were associated with BP changes after Na-load (Barlassina *et al*, 2007).

Uromodulin is the most abundant protein in urine and is specifically secreted by the TAL. The rs4293393 variant of the uromodulin gene *UMOD* was associated with increased DBP and increased response to diuretics with a better response of BP. Moreover, the

mouse transgenic line overexpressing uromodulin showed an increase in BP in a *Umod* dosage-dependent manner that could be normalized by a low NaCl diet. The salt-sensitive hypertension is correlated with abnormal activation of Na-K-Cl cotransporter (Nkcc2) (Trudu *et al*, 2013).

#### *1.2.5.3* Atrial natriuretic peptide

Atrial natriuretic peptide (ANP) reduction is correlated with salt-sensitive hypertension. KO mice for ANP show hypertension in a high salt diet and ventricular hypertrophy (Mishra *et al*, 2018). Moreover, in Dahl salt-sensitive rats, the ANP deletion increases BP, cardiac fibrosis, and reduced sodium and chloride excretion (Ilatovskaya *et al*, 2022).

#### *1.2.5.4* Klotho

Klotho is a protein mainly expressed in the kidney, but it has been identified also in other tissues. Among the various properties, Klotho inhibits the insulin-like growth factors (IGF) signaling pathway, suppresses the Wnt signaling pathway, and regulates Trpv5 calcium channels, while the Klotho-fibroblast growth factor 23 (FGF23) complex regulates phosphate absorption, mineral metabolism, and vitamin D3 expression and activity.

Low levels of circulating αKlotho are associated with aging and age-related diseases (Xu & Sun, 2015; Yamazaki *et al*, 2010), although the loss of Klotho is associated with CKD and serum levels are inversely proportional to salt-sensitivity hypertension (Yamazaki *et al*, 2010; Hu *et al*, 2011).

Zhou *et al* (2015) demonstrated that heterozygous deficient mice for Klotho gene ($KL^{+/-}$) had spontaneous BP increase that was boosted by high salt, compared to WT mice. This increased BP correlated to high salt was abolished by INCB3284, a specific CC chemokine receptor 2 (CCR2) inhibitor.

Moreover, studies on salt-sensitive hypertensive patients reveal a missense SNP (rs9536314) more associated with pressure natriuresis, with the GG and GT genotypes more representative in salt-sensitive patients. Moreover, circulating levels of Klotho were mainly related to DBP after acute salt load (Citterio *et al*, 2020).

Preclinical studies show that supplementation of rh-klotho (recombinant human klotho) decreases BP and Ang II levels and also enhanced pressure natriuresis (Takenaka *et al*, 2018).

#### *1.2.5.5* Genes linked to vascular reactivity

The solute carrier family 24 member 3 gene (*SLC24A3*) encodes for the sodium/calcium/potassium exchanger NCKX3 (Lytton, 2007). NCKX3 is a plasma membrane protein involved in maintaining intracellular calcium homeostasis (Yang *et al*, 2017). *SLC4A3* genetic variant (rs3790261) was associated with salt sensitivity of BP and with pressure natriuresis. This polymorphism could increase the cytoplasmic $Ca^{2+}$ concentration, therefore affecting the vasculature tone and BP regulation (Citterio *et al*, 2011).

The solute carrier family 8 member 1 (*SLC8A1*) encodes for the sodium/calcium exchanger NCX1, involved in the regulation of peripheral vascular resistance. It is expressed at high levels in the heart, kidney, and brain and low levels in almost all tissues (Lytton, 2007). NCX1 overexpression in mice increased BP which is further enhanced by high NaCl diets (Iwamoto *et al*, 2004). Moreover, *SLC8A1* SNPs (rs434082, rs11893826) are associated with salt sensitivity of BP (Citterio *et al*, 2011).

Protein kinase cGMP-dependent 1 (PRKG1) plays a cardiovascular function and is responsible for smooth muscle tone relaxing mediated by nitric oxide (NO) (Feil *et al*, 2002). Citterio *et al* (2011) identified three genetic variants associated with DBP variation because of acute salt load.

## 1.3 Cardiotonic steroids (CTS)

Several studies have demonstrated the CTS role in cellular function and pathophysiological process regulation (Pavlovic *et al*, 2014). Cardiotonic steroids were detected in blood plasma, adrenal gland, and hypothalamus of mammals. CTS can be divided into two distinct classes based on the five- (cardenolides) or six-membered (bufadienolides) lactone ring attached to the C17 in steroid scaffold. All CTS binds and regulates the $Na^+/K^+$-ATPase (NKA) affecting the electrochemical gradient across the plasma membrane. Endogenous CTS increase is pronounced in general volume expansion and when water and salt retention are responsible for the volume expansion in hypertension (Pavlovic *et al*, 2014).

### *1.3.1 Sodium potassium pump (NKA)*

The $N^+$-$K^+$-ATPase is a key transmembrane protein that catalyzes the active transport, by ATP hydrolysis, of 3 $Na^+$ ions outside the cells and the release of two $K^+$ ions inside the cells, against their concentration gradient across all the eukaryotic cells.

It is composed of α and β subunits and a smaller FXYD protein. The α-subunit represents the catalytic subunit and contains the binding site for ions, ATP, and CTS. On the contrary, the β subunit is responsible for enzyme regulation, localization, and stabilization of intermediates. The FXYD protein is present in some tissues, such as the kidney, heart, and brain, and modulates kinetic properties (Jorgensen *et al*, 2003).

There were four different α isoforms, α1, α2, α3, and α4, each with a specific tissues distribution. The α1 is ubiquitously expressed but it is also the predominant isoform expressed in the kidney and heart, whereas the α2 is predominantly expressed in vasculature. The α3 subunit, with a higher affinity for sodium, is most present in the brain. The α4 subunit is very divergent from the other α isoforms, with expression in very specialized cells like spermatozoa. The β subunits has also three isoforms, 1, 2, and 3, differently located among tissues (Clausen *et al*, 2017).

Due to its role in maintaining the electrochemical gradient across the plasma membrane, the NKA plays a key role in cellular homeostasis. In the kidney, the NKA activity is the driving force for $Na^+$ reabsorption that is essential for controlling volume expansion and

consequently BP. In muscle cells, the NKA activity determines the NCX versus, regulating the intracellular concentration of $Ca^{2+}$ and contractility (Pavlovic *et al*, 2014).

### *1.3.2 Endogenous ouabain*

Endogenous ouabain (EO) is a CTS belonging to the cardenolides class that was first identified in human plasma by Hamlyn *et al* in 1991. Subsequently, EO was also isolated from the bovine adrenal gland (Schneider *et al*, 1998) and hypothalamus (Kawamura *et al*, 1999).

The endogenous synthesis of EO and all CTS is still controversial because the biosynthetic pathway was not fully elucidated. Despite that, it is believed that cholesterol is the main precursor and that the synthesis is likely adrenocortical steroids. The adrenal gland was identified as the main production site of EO; thus, it was demonstrated that conscious dogs release EO from it, and an adrenalectomy experiment demonstrated a reduction in the circulating plasma level of EO (Boulanger *et al*, 1993; Masugi *et al*, 1988). Moreover, primary adrenocortical cells and rat adrenal tissue produced EO, and pregnenolone and progesterone are precursors of EO synthesis as the inhibition of progesterone abolished EO synthesis (Laredo *et al*, 1994; Perrin *et al*, 1997; Lichtstein *et al*, 1998).

The biosynthetic pathways of EO and aldosterone have a common precursor: progesterone. Their pathways start from acetil-CoA conversion to oxidosqualene that in turn is converted into lanosterol and then into cholesterol. The EO and aldosterone share biosynthesis till progesterone which is their common precursor (Figure 6) (Blaustein & Hamlyn, 2020). Tentori *et al*, (2016) demonstrated that EO and aldosterone plasma variations run together.

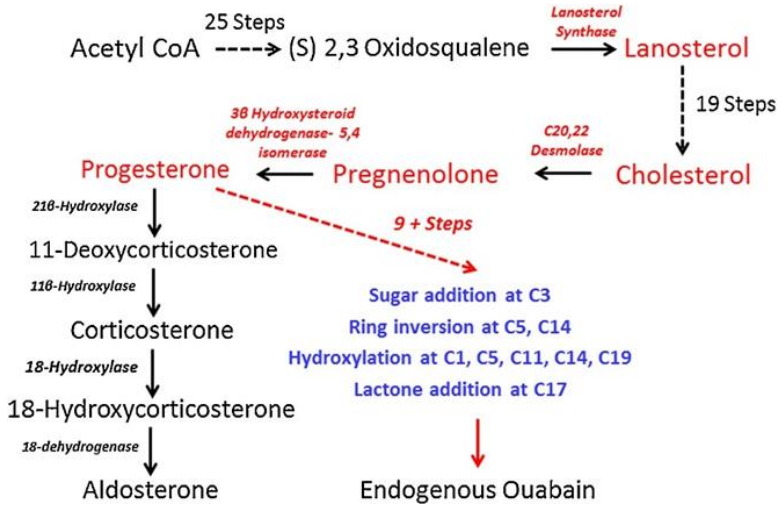


***Figure 6. Biosynthetic pathways of aldosterone and endogenous ouabain.*** *Pregnenolone and progesterone are known intermediates of EO synthesis. In red it was shown molecules demonstrated involved in EO synthesis. In blue, are steps that are involved in the EO biosynthesis but not yet demonstrated. In black, aldosterone pathway biosynthesis. Adapted from Blaustein & Hamlyn, (2020).*

### *1.3.3 EO and NKA as a membrane transporter regulator*

Cardiac glycosides are medicine, chemically analogous to endogenous CTS, used to treat congestive heart failure because of their ability to increase cardiac output. It is known for several years that the drug effect involved $Na^+$ and $K^+$ regulation (Braunwald, 1985; Page, 1964). Therefore, when CTS were identified in vertebrates, it was assumed that endogenous CTS worked as the cardiac glycosides interacting with NKA.

In this classical model, the NKA transporter activity was reduced upon CTS binding. This blocking causes an increase in the intracellular level of $Na^+$, disrupting the electrochemical gradient, thus increasing the $Ca^{2+}$ influx through NCX (Baker *et al*, 1969). The NCX is a bidirectional transporter, and the transport direction depends on plasma membrane potential: in steady state 1 $Ca^{2+}$ ion was extruded by the cells, and 3 $Na^+$ enters, but when the plasma membrane potential is affected NCX inverts its transport direction and recall $Ca^{2+}$ inside the cells. This regulation in cardiotonic effects is mostly mediated by the α2 isoform of NKA (Despa *et al*, 2012).

### *1.3.4 EO and signal transduction*

A more recent model assumed that when the circulating levels of CTSs are too low (few nM), are not able to inhibit the transporter function of NKA. Later it was demonstrated that the NKA is not only an ions transporter, but it can also act as a functional receptor and its activation is triggered by EO. NKA interacts with other proteins in the plasma membrane and regulates the protein kinase signaling cascade without affecting intracellular $Na^+$ and $K^+$ concentration (Xie & Askari, 2002).

The α isoform is responsible for the activation of different signaling cascades in the cells. Indeed, the NKA as a receptor interacts with several proteins and, the cascade activated depends on the protein which is bound to, even in the presence of the same α isoforms. The α1 subunit resides in microdomains in the plasma membrane and is involved in NKA signaling mediated by EO.

An Important pathway mediated by EO binding is the NKA-Src-EGFR signaling cascade. The NKA binding to Src maintains the protein inactive but when EO binds NKA it induces a conformational change that in turn activates Src, thus causing the releasing of EO from the NKA complex and the phosphorylation of the epidermal growth factor receptor (EGFR). This leads to the activation of different pathways such as MAPK kinase and PI-$_3$K/Akt pathway resulting in cardiac hypertrophy and metabolic dysfunction (Bagrov *et al*, 2009).

### *1.3.5 EO and hypertension*

Alterations in renal sodium management could have a key role in the pathogenesis of HTN. The $Na^+/K^+$-ATPase activity in the kidneys is regulated by hormonal factors such as EO, but also genetic determinants including ADD1 gene, associated with a higher expression of Na+/K+-ATPase in the surface of the cell and an enhancement of its activity (Tripodi *et al*, 1996).

In the Milan Hypertensive Strain (MHS), a rat model carrying the *Add1* F618Y mutation, an increased concentration of EO, with increased tubular sodium reabsorption, was detected (Ferrari *et al*, 2006). The relationship between EO and hypertensive state was also investigated by EO infusion in this rat strain showing an increase in BP, cardiac left

ventricle, and kidney and these effects could be ameliorated by rostafuroxin, an EO inhibitor (Ferrandi *et al*, 2004).

EO circulating levels and sodium balance were also examined at human level. Manunta *et al*, (2006) studied the long-term response of EO to a high-salt diet in healthy individuals. After 3 days of diet, they observed a significant increase in EO plasma circulating levels in patients on a high-salt diet compared to those on a normal salt diet. In patients with essential hypertension, an acute and chronic restriction of salt intake (but not the acute salt loading) was associated with a significant rise in EO plasmatic levels (Manunta *et al*, 2001). Moreover, under basal conditions, BPs levels are directly correlated with circulating levels of EO, thus patients with high plasma levels of EO have also high BP. After saline loading, BP did not change according to EO levels, but Na reabsorption increases only in patients with high EO levels (Manunta *et al*, 2008b). Therefore, EO is considered a positive regulator of BP during a low-salt diet, increasing BP, but preventing an increase in BP during elevated salt intake.

## 1.4 Lanosterol Synthase (LSS)

In eukaryotes, the formation of steroid scaffold is catalyzed only by oxidosqualene cyclase (OSC; EC 5.4.99.7), commonly known as Lanosterol synthase, a membrane protein associated with the cytosolic side of the endoplasmic reticulum, that converts the linear 2,3-oxidosqualene into lanosterol. Lanosterol is a tetracyclic triterpenoid and represents the steroid scaffold from which are derived all steroids in higher organisms (Thoma *et al*, 2004).

The interest in OSC has increased over years as a possible therapeutic target alternative to statin treatment. Lanosterol synthase inhibition not only reduces the lanosterol formation but also increases 24(S),25-epoxycholesterol synthesis, an oxysterol that inhibits HMG-CoA reductase activity, resulting in cholesterol-lowering (Mark *et al*, 1996).

Lanosterol synthase is encoded by the *LSS* gene localized on the 21q22.3 locus in humans (Ensembl: ENSG00000160285 ) (Young *et al*, 1996) and 10 C1; 10 39.1 cM in mice (Ensembl: ENSMUSG00000033105). The *LSS* has 11 transcripts in humans, and it is expressed predominantly in the skin, lung, liver, and adrenal gland but also in the kidney and other tissues (Fagerberg *et al*, 2014; Lee *et al*, 2015).

The protein is conserved in eukaryotes and is produced also by few prokaryotes (Lamb *et al*, 2007). It is 732 aa long in *H. sapiens* ( NCBI reference sequence: NP_001001438) and 733 in *M. Musculus* (NCBI reference sequence: NP_666118) with a homology of 86% between them calculated by SIM (Alignment Tool for protein sequences; Expasy). LSS consists of two (α/α) barrel domains and the active-site cavity is located in between.

### *1.4.1 Allelic variants of LSS gene*

The primary structure of the enzyme has several natural variations and mutations. Mori *et al*, (2006) identified mutations in the *Lss* gene associated with cataracts in the Shumiya cataract rat (SCR), a hereditary cataractous strain. These mutations, in addition to the one identified in *Fdft1*, cause a cholesterol reduction in the lens and therefore are responsible for cataracts in this rat strain. Other mutations in highly conserved amino acids in LSS gene were identified associated with congenital cataracts (Chen & Liu, 2017; Zhao *et al*, 2015). G588S and W581R mutations were identified by screening families affected by

severe congenital cataracts. Both mutations reduce lanosterol, without affecting cholesterol production, which plays a key role in cataract formation (Zhao *et al*, 2015).
LSS mutations were also identified, by whole-exome sequencing, associated with autosomal-recessive hypotrichosis simplex (HS), a rare disorder characterized by scalp and body hair loss. Romano *et al*, (2018) identified in patients with scalp hair at birth, four missense and one nonsense biallelic mutations of *LSS* gene that affect highly conserved residues resulting in a wrong localization of the protein in the cytoplasm.
Other variants were identified by studying a neuroectodermal syndrome named alopecia with mental retardation syndrome (APMR). All the biallelic pathogenic variants identified span the entire *LSS* gene and are not associated with plasma cholesterol impairment (Besnard *et al*, 2019).
In our laboratory, we identified an interesting variant in *LSS* gene, the rs2254524, associated with higher BP in adolescents (Bigazzi *et al*, 2020), a faster decline of BP after a low salt diet (Lanzani *et al*, 2016), and associated with accelerated eGFR in a prospective hypertensive cohort and with high risk for AKI in patients before cardiovascular surgery (Iatrino *et al*, 2019).

### *1.4.2 LSS rs2254524 SNP*

The interest of our laboratory in the LSS rs2254524 began in 2010 when Lanzani *et al*, (2010) started studying genes involved in the endogenous ouabain synthesis and identified *a priori* specific genetic variants that could be used as plausible candidates predicting the rostafuroxin pharmacological response in naive essential hypertensive (NHE) patients.

They chose the *LSS* rs2254524 SNP as a candidate gene because of its possible role in the endogenous ouabain and all steroid biosynthesis. Indeed, the rs2254524 SNP is a missense variation localized in the exon 20 of the *LSS* gene responsible for a missense mutation that causes the substitution of the Valine 642 (CC=Val; wild variant) in a Leucine (AA=Leu; minor variant) (NM_002340.6: c.1924T>G; L [TTG] > V [GTG]). The minor allele frequency (MAF) of the A allele in the Caucasian general population is 33% (1000 Genomes Project - phase 3).

In the human kidney, the *LSS* mRNA level was reduced in the LSS AA mutant samples compared to the WT (0.67±0.34 vs 1.03±0.23 arbitrary unit; $p$=0.026). Moreover, adrenocortical cell lines (H295R) transfected with the A variant of *LSS* show a reduced *LSS* mRNA and protein level compared to that transfected with the C variant (Figure 7) (Lanzani *et al*, 2010). LSS activity and ouabain production were higher in both wild-type and mutant H295R cells compared to the mock, but when normalized for the LSS protein expression, both LSS activity and ouabain production become higher only in mutant cells compared to the wild-type (Figure 7) (Lanzani *et al*, 2010).

Moreover, this work also demonstrated that rostafuroxin did not show a statistically significant fall in SBP compared to the placebo in the overall cohort. However, the SBP response changed if we considered the data stratified based on the genotypes. *LSS* rs2254524 was associated with a higher reduction of SBP after rostafuroxin treatment (Lanzani *et al*, 2010).

Furthermore, it was demonstrated that the rs2254524 variant was associated with a higher level of SBP and DBP in adolescents carrying the A alleles compared to the one carrying the C one (Bigazzi *et al*, 2020).

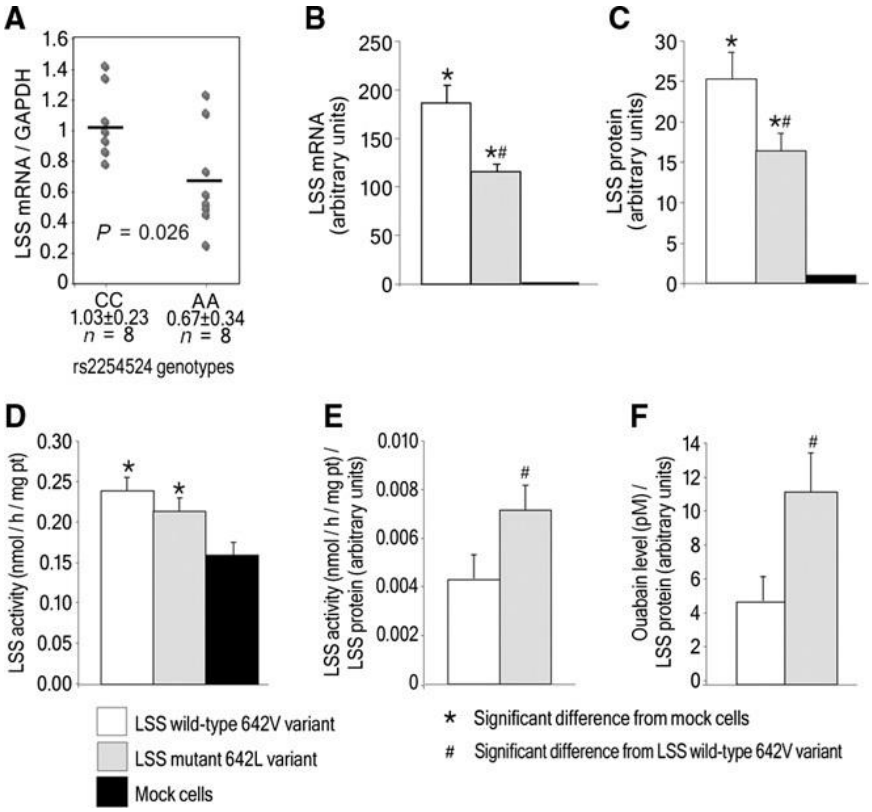


***Figure 7. Expression analysis of LSS polymorphism in human kidneys and H295R-transfected cells.*** *(**A**) LSS mRNA expression in renal cortex. (**B**) LSS mRNA levels in transfected cells. Data are expressed as arbitrary units relative to the mock level. (**C**) LSS protein levels in transfected cells. (**D**) LSS activity in transfected cells was expressed as micromoles of [$^{14}$C]acetate incorporated in lanosterol per hour per milligram. (**E**) The ratio between LSS activity and LSS protein. (**F**) The plot shows the ratio between ouabain secretion and LSS protein. From Lanzani et al, (2010).*

### *1.4.3 LSS rs2254524 and salt sensitivity*

Endogenous ouabain circulation is regulated by $Na^+$ balance: long-term high-salt diets suppress EO levels, whereas $Na^+$ depletion stimulates EO production. Therefore, Lanzani *et al*, (2016) analyzed the response of SBP and DBP after a low salt diet in NHE patients carrying the AA, wild-type CC, and AC variants. All the patients enrolled underwent the low salt diet protocol for 4 weeks and BP measurement, blood, and 14-hour urine samples were collected at enrolment, in half, and at the end of the protocol. The compliant patients, who completed the dietary period, carrying the AA genotype of the LSS showed a greater reduction in SBP and DBP compared to AC and CC patients, and only CC and AC patients show an increase in plasma EO after the low sodium diet (Figure 8A).

Moreover, the pressure natriuresis analysis revealed a different relationship and a less steep slope in LSS AA patients compared to LSS AC and CC, indicating an increase in salt-sensitivity of SBP to salt changing with the diet (Figure 8B) (Lanzani *et al*, 2016).

Intriguingly, in yeast triterpenoids are biosynthesized from 2,3-oxidosqualende by the OSC Lanosterol synthase. Induction of OSCs gene expression in *Saccharomyces cerevisiae* deficient for Lanosterol synthase (GIL77) weakened their tolerance to salt stress since triterpenoids accumulated in the plasma membrane (Inafuku *et al*, 2018).

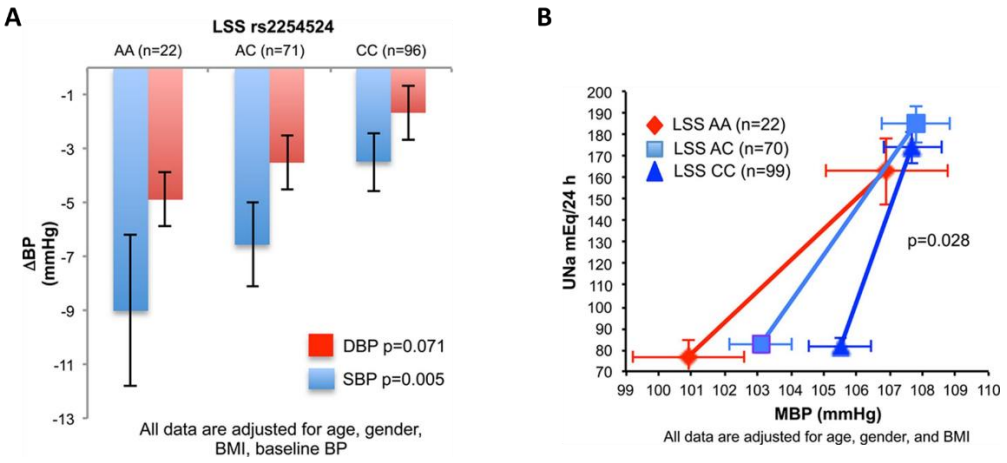


***Figure 8. SBP and renal pressure natriuresis in patients during low salt diet A.*** *ΔBP represents the decline in SBP and DBP during the low salt diet.* ***B.*** *Renal pressure-natriuresis relationship analysis. Una is the urinary sodium excretion and MBP is the mean blood pressure. From Lanzani et al, (2016).*

### *1.4.4 LSS rs2254524 in acute kidney injury (AKI) and chronic kidney disease (CKD)*

An association study between the LSS rs2254524 variant and the postoperative AKI developed in patients undergoing cardiovascular surgery revealed a significantly higher association between AA patients and the incidence of AKI after surgery. Moreover, a follow-up for a median of four years in a longitudinal study of patients with essential hypertension, demonstrated that individuals carrying the A allele had a higher risk of eGFR faster decline (Iatrino *et al*, 2019).

AA patients analyzed in both these cohorts did not differ from CC individuals in plasma circulating level of EO, but only in the kidney amount of EO that is higher in AA compared to CC human nephrectomies, in both cortex and medulla (Iatrino *et al*, 2019), therefore indicating the potential value of LSS rs2254524 genotype–based risk

stratification to identify patients at high risk for predisposition to develop AKI and for the progression of eGFR decline in the natural history of CKD.

## 2. AIM OF THE WORK

Several association studies performed in hypertensive patients have identified genes and their variants (SNP) as associated with hypertension onset or progression. Then, the new technology and the availability of an easier way to generate a new mouse model could help in the identification of the causal link between these genes and their SNPs in the pathogenesis of hypertension and salt sensitivity.

EO is a CTS that binds the NKA and blocks the $Na^+$ excretion that causes an increase in intracellular $Ca^{2+}$ through NCX1 or activates the Src-EGFR pathway. This action increases vasculature contractility, blocks kidney $Na^+$ excretion, and is responsible for salt sensitivity. EO circulating levels in hypertensive patients are higher than in normotensives. On the other hand, at low non-inhibitory concentrations, EO functions as a hormone that triggers intracellular signaling pathways. Moreover, rats infused with ouabain showed an increase in SBP and other hallmarks of hypertensive diseases, such as kidney dysfunction and increased cardiac left ventricle.

Therefore, we can speculate that increased production of EO is responsible for hypertensive onset. Even though the sequencing pathway of EO was not fully elucidated, it was demonstrated that pregnenolone and progesterone are precursors of EO. Thus, our group previously selected *a priori* SNPs in genes coding for enzymes involved in steroid biosynthesis as possible regulators of EO levels. The most interesting gene identified was *LSS* and specifically the *LSS* rs2254524 SNP. The patients carrying the minor allele variant were associated with salt-sensitive hypertension and had an increased circulating level of plasma EO after a low-salt diet.

In this study, we specifically aimed at investigating the causal correlation between missense variation rs2254524 in the *LSS* gene, the production of EO, and the possible salt-sensitive hypertensive onset in a new *Lss* knock-in (KI) mouse model carrying this informative SNP observed in humans. The experimental plan regarded firstly the basal characterization of the *Lss* KI mice, then proceeding with the evaluation of the effect of this mutation on kidney functionality and BP regulation at baseline and after different salt intake, and on renal ions and water handling, on mRNA expression at the different organs level, on the renal transcriptome, and EO.

# 3. RESULTS

## 3.1 *Lss* V643L Knock-in mouse generation

To test our hypothesis, we generated a knock-in mouse model (*Lss* V643L KI) carrying the missense mutation homologous to the human rs2254524 SNP (V642L) identified in hypertensive patients.

In collaboration with the Core Facility for Conditional Mutagenesis (CFCM) at San Raffaele Scientific Institute, we introduced by CRISPR-Cas9 technology a single nucleotide mutation (**G**TG in **T**TG), causing the substitution of a Valine in a Leucine at position 643 (V643L) in the exon 20 of *Lss* gene (Figure 9A).

We first try to microinject CRISPR-Cas9 directly in the zygote, but it had never been given KI mice. Therefore, we moved to embryonic stem cell manipulation, and we screened more than 500 clones before identification of the proper clone from which we generated the *Lss* KI mouse model used in this study.

We promoted the Homology Directed Repair (HDR) in ESCs by using donor oligonucleotide with homology arms to the target sequence. To increase the accuracy of homology-directed repair, the donor oligonucleotide had an additional silent PAM-blocking mutation (protospacer adjacent motif) (Figure 9A). Positive clones were tested by Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) analysis. The wild-type clones were recognized by the BsgI restriction enzyme resulting in 569 bp and 96 bp fragments, whereas in the presence of *Lss* V643L mutation, the recognition site and the cutting were abolished, and the gel reveals an additional fragment of 665 bp (Fig. 1B).

The transmission of *Lss* V643L mutation in the germline was tested by Allele-specific oligonucleotide-PCR (ASO-PCR) that can discriminate WT *Lss* and mutant alleles by a common forward primer and an allele-specific reverse primer (Figure 9C). Moreover, DNA sequencing confirmed the proper insertion of mutation in mice (Figure 9D).

By intercrossing heterozygous $Lss^{WT/V643L}$ mice, we got offspring expressing all three genotypes ($Lss^{WT/WT}$, $Lss^{WT/V643L}$, $Lss^{V643L/V643L}$).

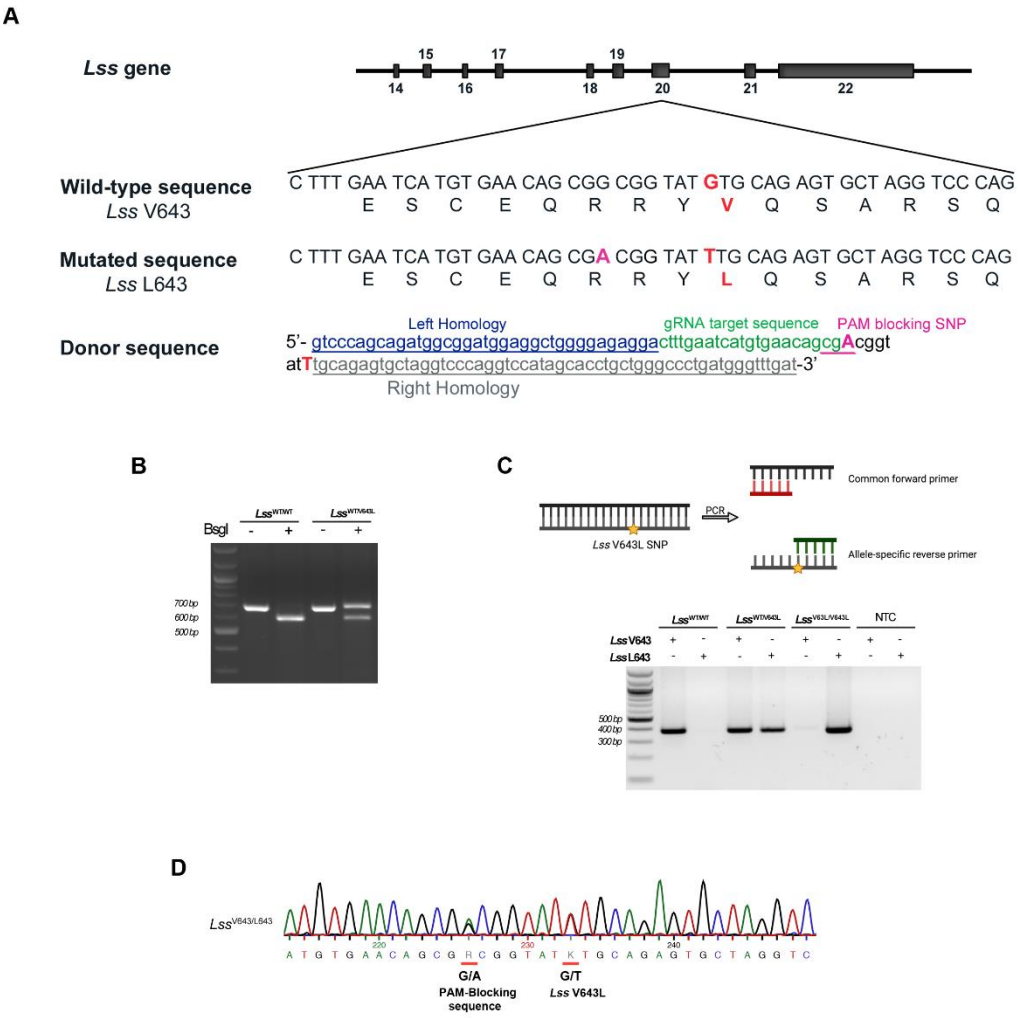


***Figure 9*. *Generation of Lss V643L knock-in mice. A.*** *Scheme of the Lss gene in conjunction with the wild type and mutated nucleotide sequences, with the corresponding amino acid sequence given below nucleotides. The V643L substitution is shown in red, and the PAM-blocking mutation is in pink. The donor sequence marked the two homology arms underlined in blue (left) and grey (right), the gRNA target sequence is reported in green and the PAM sequence in pink, and the PAM-blocking mutation is highlighted in pink.* ***B.*** *RFLP screening of* $Lss^{WT/WT}$ *and* $Lss^{V643L/V643L}$ *ESCs clones by 2 % agarose gel electrophoresis. BsgI recognized the restriction site in the WT clone, generating two RFLP fragments of 569 bp and 96 bp. The insertion of mutation deleted the restriction site generating a fragment 665 bp long.* ***C.*** *Allele-specific PCR scheme and the amplicons analysis by 2 % agarose gel electrophoresis.* ***D.*** *Sanger sequencing of heterozygous mice. The V643L and PAM-blocking sequences are underlined in red.*

## 3.2 Basal Characterization of *Lss* knock-in mice

We performed the basal characterization of male and female *Lss* V643L KI mice at 3 and 12 months. For reasons of simplification, we reported in the main figure (Figure 10) only results obtained in male mice since a comparison between genotyping reveals no differences in most of the analyzed parameters within the group based on sex, except for the kidney weight normalized on the body weight at 3 and 12 months of age. Basal characterization of female mice at 3 and 12 months is reported in the appendix section (Appx Table 1).

There were no differences in the body weight in *Lss*$^{V643L/V643L}$ homozygous mutant mice compared to *Lss*$^{WT/WT}$ mice up to 12 months of age. Accordingly, homozygous mice are healthy and phenotypically indistinguishable from their WT counterparts (Fig. 10A).

However, the biometric measures of organs at 3 months of age revealed that kidney weight normalized on body weight was slightly increased in *Lss*$^{V643L/V643L}$ mice compared to WT mice, differently from all other organs analyzed that did not show any differences in mass. At 12 months of age, this increasing trend is conserved in the kidney and emerged also in the liver of *Lss*$^{V643L/V643L}$ mice compared to WT (Fig. 10B).

Lanosterol synthase catalyzes the formation of Lanosterol, the starting compound for all steroid hormone biosynthesis, including cholesterol synthesis. Hepatic cells produced the greatest amount of cholesterol in the body, while EO is produced in the adrenal cortex. Therefore, we assessed the impact of V643L mutation at mRNA levels in the adrenal gland and liver, in addition to the kidney and heart selected for their role in the regulation of BP. At 3 and 12 months of age, the V643L mutation did not affect the mRNA level of *Lss* in all organs in mutant mice compared to the WT counterpart (Fig. 10C).

Furthermore, no differences were detected in the circulating level of cholesterol between WT (99.09 ±12.9 mg/dl) and LSS$^{V643L/V643L}$ mice (96.66 ± 15 mg/dl) (Table 1).

In conclusion, the V643L mutation does not affect mice phenotypically and cholesterol production but increases kidney and liver weight without impact on *Lss* mRNA levels.

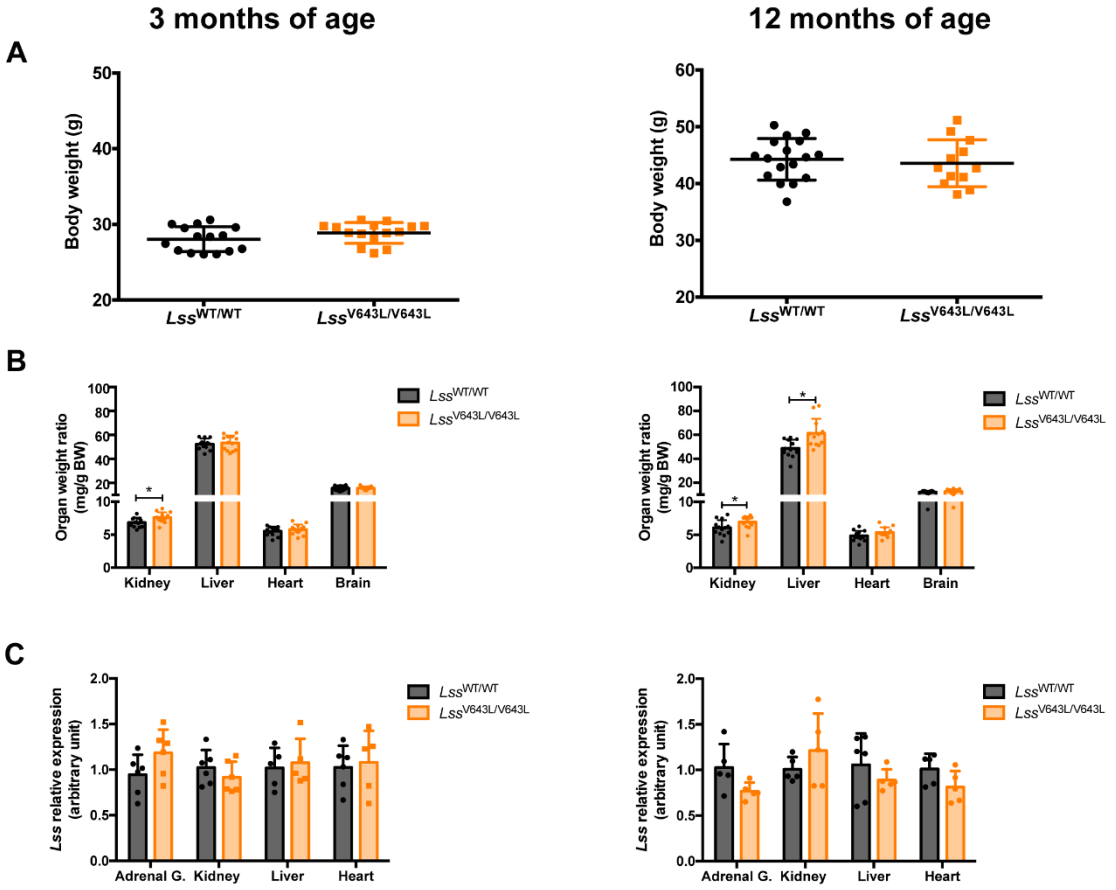


***Figure 10. Basal characterization of Lss$^{V643L/V643L}$ KI mice at 3 and 12 months of age. A.*** *Body weight measurement in Lss$^{V643L/V643L}$ mice compared to Lss$^{WT/WT}$ at 3 (n =15) and 12 (n = 12-17) months of age.* ***B.*** *Adrenal gland, kidney, liver, and heart weight normalized to body weight measured in mice at 3 and 12 months of age (n = 10-13). Statistical significance was calculated by unpaired student's t-test (* p<0.05).* ***C.*** *Bar charts represent the Lss mRNA levels relative to the Gapdh (ΔCt) and normalized to the average ΔCt values of Lss$^{WT/WT}$ mice for each organ (n = 5). Data are shown as mean ± standard deviation (SD).*

## 3.3 Effects of *Lss* V643L mutation on kidney functionality and BP regulation at baseline

Since the results obtained in hypertensive patients about the role of LSS in AKI (Iatrino *et al*, 2019) and the increase in kidney body mass observed in mice, we first assessed the kidney functionality by biochemical measurements at baseline. We specifically analyzed creatinine clearance and blood urea nitrogen (BUN) in WT and mutant mice, 3 and 12 months old. V643L mutation in mice did not significantly affect creatinine in serum and urine. Moreover, no difference was detected in urea content in serum (Table 1). Female data at 3 months of age are reported in the appendix section (Appx Table 2).

Furthermore, we assessed the SBP level by the tail-cuff system every two days for ten days. On the day of the SBP recording, we performed fifteen measurements, the first five were considered as acclimation for the mice and useless, and the last 10 were used for the analysis (Figure 11A). We did not observe differences in the SBP levels at the selected time points and both genotypes (Figure 11B).

Finally, EO quantification levels in the kidney of mice at both 3 and 12 months of age at basal level did not reveal any statistical difference between the $Lss^{V643L/V643L}$ (1.267 ± 0.31 pg/mg kidney; $n$=7) and $Lss^{WT/WT}$ mice (1.492 ± 0.34 pg/mg kidney; $n$=7).

**Table 1. Serum and urine biochemical analysis**

| | 3 months | | 12 months | |
|---|---|---|---|---|
| | ***Lss*$^{WT/WT}$** | ***Lss*$^{V643L/V643L}$** | ***Lss*$^{WT/WT}$** | ***Lss*$^{V643L/V643L}$** |
| **Serum** | | | | |
| Creatinine (mg/dL) | 0.38 ± 0.05 | 0.34 ± 0.05 | 0.41 ± 0.02 | 0.36 ± 0.11 |
| Cholesterol (mg/dL) | 98.33 ± 10.02 | 97.57 ± 10.75 | 112.8 ± 8.22 | 97.33 ± 19.15 |
| Urea (BUN) | 53.67 ± 9.89 | 51.11 ± 8.35 | 50.88 ± 7.99 | 51.38 ± 11.5 |
| **Urine** | | | | |
| Diuresis (µl) | 1.16 ± 0.31 | 0.98 ± 0.42 | 1.91 ± 1.08 | 2.83 ± 1.88 |
| Urine Flow Rate (µl/min/g) | 0.04 ± 0.011 | 0.03 ± 0.014 | 0.04 ± 0.02 | 0.07 ± 0.05 |
| Creatinine (mg/dL) | 31.01 ± 8.78 | 39.8 ± 10.61 | 34.9 ± 15.0 | 33.0 ± 11.5 |
| $Na^+$/Crea (mM/mg) | 0.52 ± 0.05 | 0.54 ± 0.09 | 0.43 ± 0.13 | 0.47 ± 0.13 |
| $Cl^-$ /Crea (mM/mg) | 0.62 ± 0.04 | 0.63 ± 0.10 | 0.45 ± 0.13 | 0.49 ± 0.11 |
| $K^+$ /Crea (mM/mg) | 0.89 ± 0.05 | 0.92 ± 0.14 | 0.75 ± 0.17 | 0.76 ± 0.07 |
| Creatinine Clearance (µl/min/g) | 3.05 ± 1.49 | 3.34 ± 1.41 | 3.65 ± 1.36 | 4.91 ± 2.93 |

Data are expressed as mean ± SD; *n* = 6-11

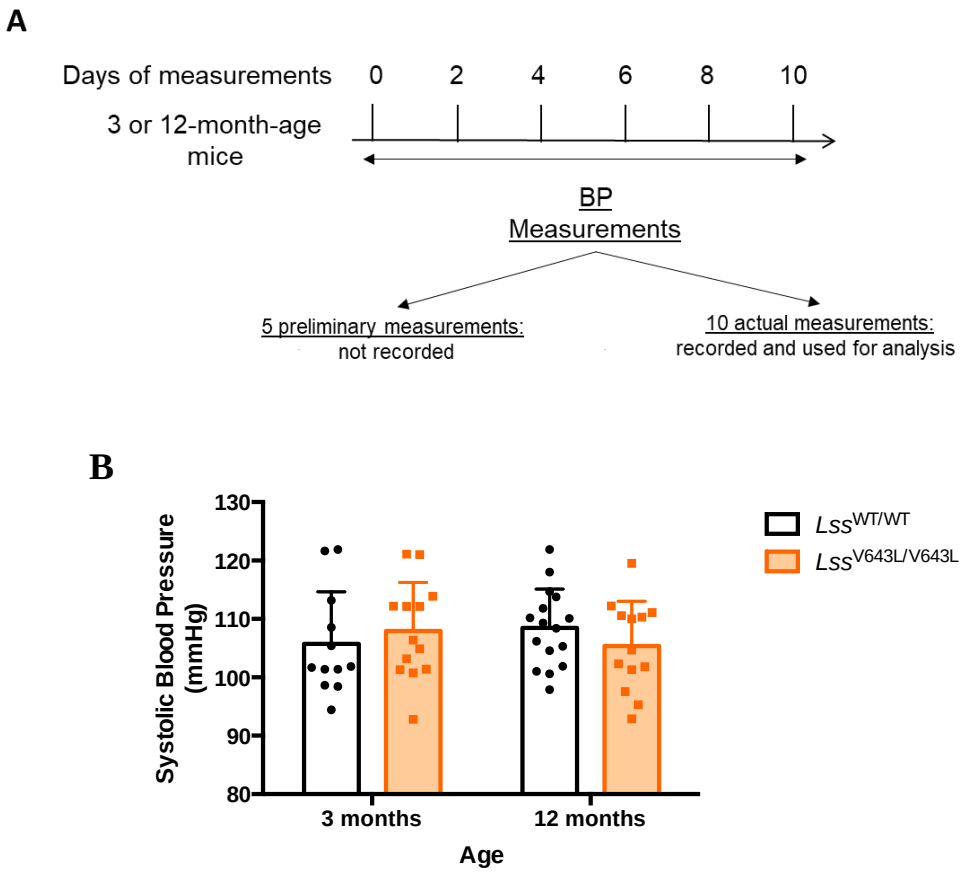


***Figure 11. Measure of systolic BP basal level in Lss$^{WT/WT}$ and Lss$^{V643l/V643L}$ mice. A.*** *SBP was measured in all mice for 10 days. We performed 15 times recording with first 5 preliminary measurements not recorded and 10 measurements that was used for the analysis, per day of SBP recording.* ***B.*** *The bar chart represents the 10$^{th}$ day of SBP measurements in mice under standard chow. For proper mouse training, SBP was measured by the tail-cuff method at the age of 3 and 12 months every 2 days for 10 days as in the scheme in A (n = 12-16). The data are represented as the mean value of at least 5 out of 10 measurements per mouse per day of measuring. Data are shown as mean ± SD.*

## 3.4 *Lss* V643L variant and the systolic BP responsiveness to different salt intake

In patients, the LSS A variant was already associated with a higher reduction in SBP and DBP, compared to the C variant, upon a low salt diet protocol (Lanzani *et al*, 2016). Hence, we tested the response of *Lss* mice to different salt concentrations administered by diet. $Lss^{V643L/V643L}$ and $Lss^{WT/WT}$ mice were divided into 3 groups each fed with a different diet at variable salt concentration: control diet (NSD; 0.5 % NaCl), high salt diet (HSD; 4 % NaCl) and low salt diet (LSD; <0.03 % Na) for a total of about 3 weeks before sacrifice (Figure 12A).

First, SBP response to salt intake at 3 months of age did not show any differences after 10 days of a high and low salt diet, in both genotypes (Figure 12B).

The salt sensitivity of SBP affects 40-50% of adult hypertensive patients, but these percentages rise with the increase in age. Therefore, we measured the SBP in mice at 12 months of age and we observed an increase of SBP after the diet at high content of NaCl in $Lss^{V643L/V643L}$ mice compared to their WT counterparts (Figure 12B). $Lss^{WT/WT}$ mice did not show any significant variation in SBP upon diet treatment (Figure 12C).

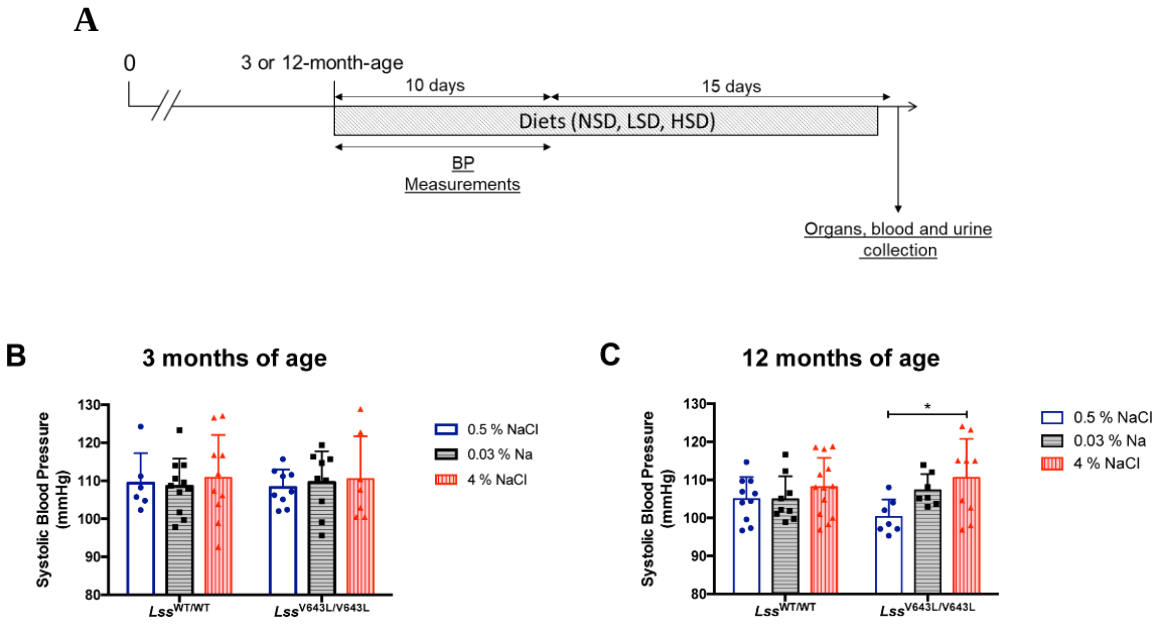


***Figure 12. SBP measurement in Lss mice after different salt intake. A.*** *Schematic representation of protocols used during diet treatment. Briefly, mice at 3 or 12 months of age started the diet protocol and BP measurements concomitantly. At the end of 10 days, we measured the SBP. After metabolic cage training, we collected blood and urine and sacrificed mice for organ harvesting.* ***B.*** *SBP measurements by tail-cuff methods in* $Lss^{V643L/V643L}$ *and* $Lss^{WT/WT}$ *mice at 3 months of age, after 10 days of control (0.5 % NaCl; blue bar), low-salt (0.03 % Na; grey striped bar) and high-salt (4 % NaCl; red striped bar) diets. Bars are mean ± standard deviation (n = 6-10).* ***C.*** *SBP measurements by tail-cuff methods in* $Lss^{V643L/V643L}$ *and* $Lss^{WT/WT}$ *mice at 12 months of age; all the characteristics of SBP measurement and analysis were maintained, as explained in A (n = 7-13). Statistical analysis was performed by two-way ANOVA (p < 0.05 %).*

## 3.5 Effect of different diets on renal ions and water handling

To assess the possible effects of diets with different sodium content on renal function, ions, and water handling, we collected urine and serum from 12-month-old mice upon 25 days of high salt and control diets.

With an equal food intake, there is an increased trend in water intake in mice under a high-salt diet compared to the control diet in both genotypes, according to NaCl diet content (Figure 13A and Table 2).

Moreover, a decreased urine flow rate (UFR) was observed in *Lss*$^{V643L/V643L}$ mice compared with WT counterparts, when both genotypes were fed with HSD (Figure 13B).

As expected, high-salt diets also induced an increased urine flow rate in mice in high-salt diets, followed by an increased $Na^+$ and $Cl^-$ excretion (Figures 13C and 13D).

Moreover, the amount of urinary $K^+$, both absolute and normalized on urine creatinine, was increased in *Lss*$^{V643L/V643L}$ mice in the high-salt diet compared to mutant mice in the control diet and compared to WT mice in high-salt diet (Figure 13E).

We also observed a statistically significant decreased level of serum $Cl^-$ in *Lss*$^{V643L/V643L}$ mice compared to *Lss*$^{WT/WT}$ (Table 2).

*Lss*$^{WT/WT}$ mice in HSD showed an increase level of urea compared to *Lss*$^{WT/WT}$ in NSD.

Endogenous ouabain measurements in total kidney revealed any differences in the EO level in both genotypes after salt protocols (Figure 13F).

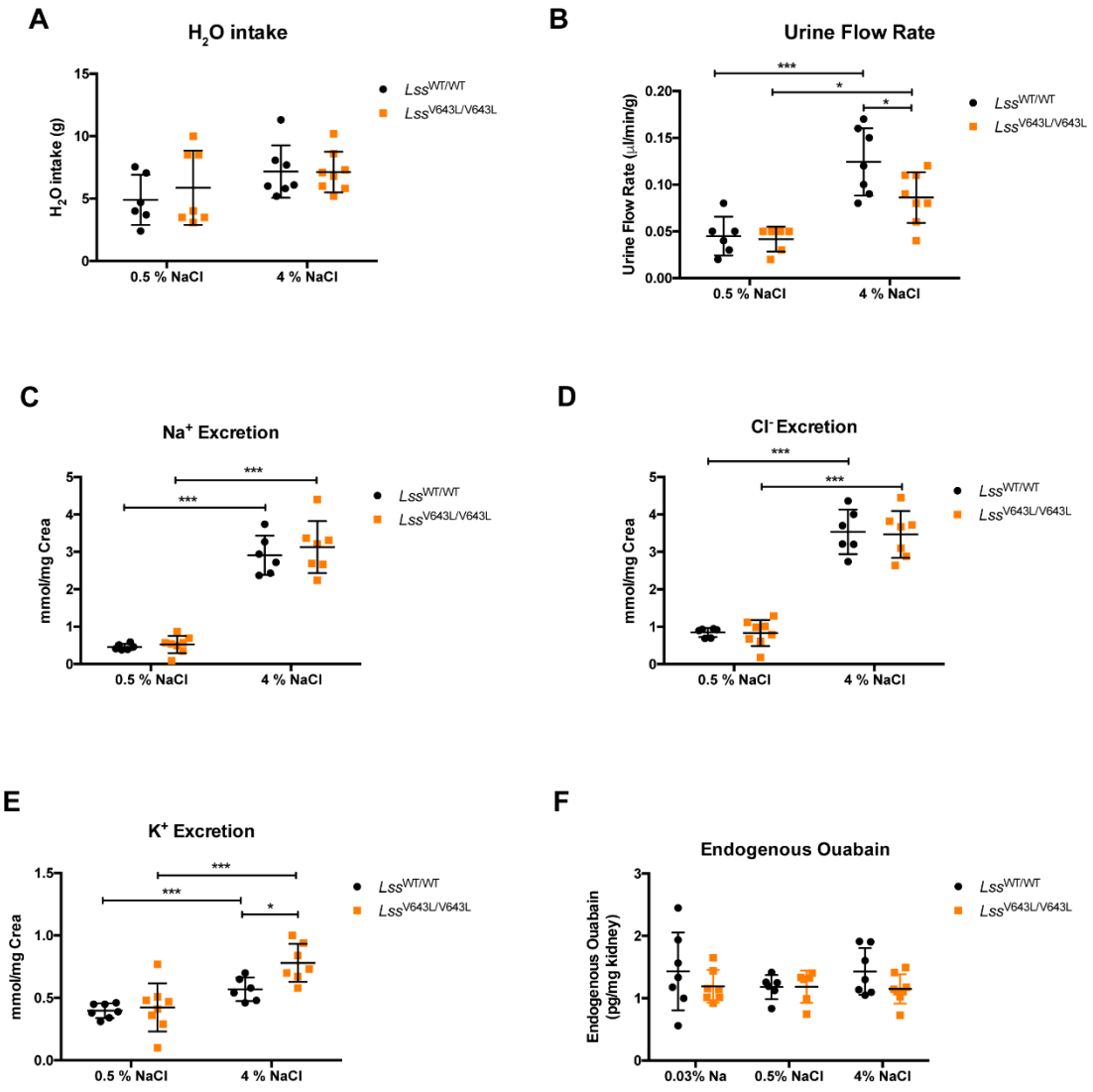


***Figure 13. Analysis of different salt intake on water and ions excretion. A.*** *$H_2O$ intake for 16-hour-measurement in the metabolic cage expressed in grams.* ***B.*** *Urine Flow Rate (UFR) is reported as µl of urine per minute per body weight measured in grams.* ***C.*** *$Na^+$ urinary excretion normalized on creatinine expressed in mg excreted in the urine.* ***D.*** *$Cl^-$ urinary excretion normalized on urine creatinine.* ***E.*** *$K^+$ urinary excretion normalized on urine creatinine excretion.* ***F.*** *Endogenous ouabain measures by RIA assay in the three different diet conditions in WT and mutant mice. Statistical analysis performed by two-way ANOVA (*p<0.05; **P>0.01; ***p<0.001*

**Table 2. Biochemical analysis of $Lss^{WT/WT}$ and $Lss^{V643L/V643L}$ mice at 12 months of age under control and high-salt diets.**

| | 0.5 % NaCl | | 4 % NaCl | | *p-value* |
|---|---|---|---|---|---|
| **Parameter** | ***Lss*$^{WT/WT}$** | ***Lss*$^{V643L/V643L}$** | ***Lss*$^{WT/WT}$** | ***Lss*$^{V643L/V643L}$** | |
| Water Intake (g) | 4.90 ± 1.83 | 5.87 ± 2.76 | 7.17 ± 1.94 | 7.13 ± 1.52 | ns |
| **Serum** | | | | | |
| Creatinine (mg/dL) | 0.39 ± 0.16 | 0.29 ± 0.04 | 0.40 ± 0.10 | 0.34 ± 0.12 | ns |
| Urea (mg/dL) | 50.88 ± 7.47 | 51.38 ± 10.76 | 61.38 ± 9.53 | 57.00 ± 10.21 | <0.05 * |
| $Na^+$ (mmol/L) | 166.29 ± 2.81 | 166.7 ± 11.05 | 166.8 ± 2.59 | 166.8 ± 4.15 | ns |
| $Cl^-$ (mmol/L) | 121.5 ± 3.46 | 117.4 ± 2.12 | 122.0 ± 2.73 | 119.8 ± 2.82 | <0.05 $ |
| $K^+$ (mmol/L) | 5.54 ± 0.81 | 4.47 ± 1.09 | 5.96 ± 0.47 | 5.41 ± 0.68 | ns |
| **Urine** | | | | | |
| Urine (ml/16h) | 2.79 ± 1.76 | 2.43 ± 1.67 | 4.22 ± 1.37 | 3.45 ± 1.11 | ns |
| Urine Flow Rate (µl/min/g) | 0.05 ± 0.02 | 0.04 ± 0.01 | 0.12 ± 0.03 | 0.09 ± 0.02 | <0.001 * + <0.05 # |
| Creatinine (mg/dL) | 25.78 ± 13.03 | 36.14 ± 11.05 | 15.17 ± 4.17 | 16.99 ± 2.61 | <0.01 + |
| Proteins | 177.1 ± 47.9 | 208.3 ± 22.42 | 166.3 ± 44.3 | 167. 0 ± 22.64 | <0.05 + |
| Electrolytes | | | | | |
| $Na^+$ (mmol/L) | 119.6 ± 44.39 | 143.78 ± 70.89 | 456.63 ± 153.19 | 519 ± 68.15 | <0.001 * + |
| $Cl^-$ (mmol/L) | 217.88 ± 80.93 | 233.13 ± 114.7 | 523.13 ± 116.23 | 576.14 ± 49.4 | <0.001 * + |
| $K^+$ (mmol/L) | 102.24 ± 51.03 | 116.5 ± 54.18 | 84.64 ± 23.6 | 131.76 ± 27.92 | <0.05 # |
| $Na^+$/Crea (mM/mg) | 0.52 ± 0.14 | 0.52 ± 0.23 | 2.77 ± 0.60 | 3.13 ± 0.70 | <0.001 * + |
| $Cl^-$/Crea (mM/mg) | 0.93 ± 0.19 | 0.83 ± 0.35 | 3.53 ± 0.60 | 3.50 ± 0.63 | <0.001 * + |
| $K^+$/Crea (mM/mg) | 0.41 ± 0.07 | 0.42 ± 0.19 | 0.53 ± 0.13 | 0.78 ± 0.15 | <0.001 * + <0.05 # |
| Creatinine Clearance (µl/min/g) | 4.24 ± 2.0 | 5.54 ± 1.71 | 5.22 ± 1.10 | 4.67 ± 1.83 | ns |

Data are mean ± SD.

Statistical significances relationship:

* *Lss*$^{WT/WT}$ 0.5 % NaCl vs *Lss*$^{WT/WT}$ 4 % NaCl

\+ *Lss*$^{V643L/V643L}$ 0.5 % NaCl vs *Lss*$^{V643L/V643L}$ 4 % NaCl

\# *Lss*$^{WT/WT}$ 4 % NaCl vs *Lss*$^{V643L/V643L}$ 4 % NaCl

$ *Lss*$^{WT/WT}$ 0.5 % NaCl vs *Lss*$^{V643L/V643L}$ 0.5 % NaCl

## 3.6 High salt intake effects on different organs and mRNA expression in *Lss*$^{V643L/V643L}$ mice

Hypertension is one of the leading causes of cardiovascular diseases (CV), hence we investigated hypertension-mediated organ damage (HMOD), a marker of CV, in the heart and kidney of mice 12 months old under an HSD and NSD.

Heart postmortem analysis suggested a hypertrophic phenotype in *Lss*$^{V643L/V643L}$ mice treated with a high-salt diet. Indeed, heart weight normalized to tibial length (HW/TL) measurements revealed an enlargement of the heart in *Lss*$^{V643L/V643L}$ mice under HSD, compared to *Lss*$^{V643L/V643L}$ mice under NSD, and this difference was not observed in WT mice (Fig. 14A). Moreover, histological analysis of heart morphology reveals a slight increase in the incidence of fibrosis with few inflammatory mononuclear cells in Lss$^{V643L/V643L}$ mice compared to the WT counterpart under the HSD (Figure 14B).

Instead, the kidney was not morphologically affected by the salt protocol, as we did not detect any differences between genotypes in creatinine, urea, and urinary protein levels (Table 2). Furthermore, we did not detect any structural changes by histological analysis in the kidney (data not shown).

We examined if the mRNA expression of *Lss* is influenced by salt intake by analysis of its expression in different tissues. In the adrenal gland and liver, *Lss* mRNA expression was reduced in *Lss*$^{V643L/V643L}$ mice compared to *Lss*$^{WT/WT}$ mice under HSD. In contrast, *Lss* mRNA was reduced in heart of *Lss*$^{V643L/V643L}$ mice compared to *Lss*$^{WT/WT}$ mice in HSD (Fig. 14C).

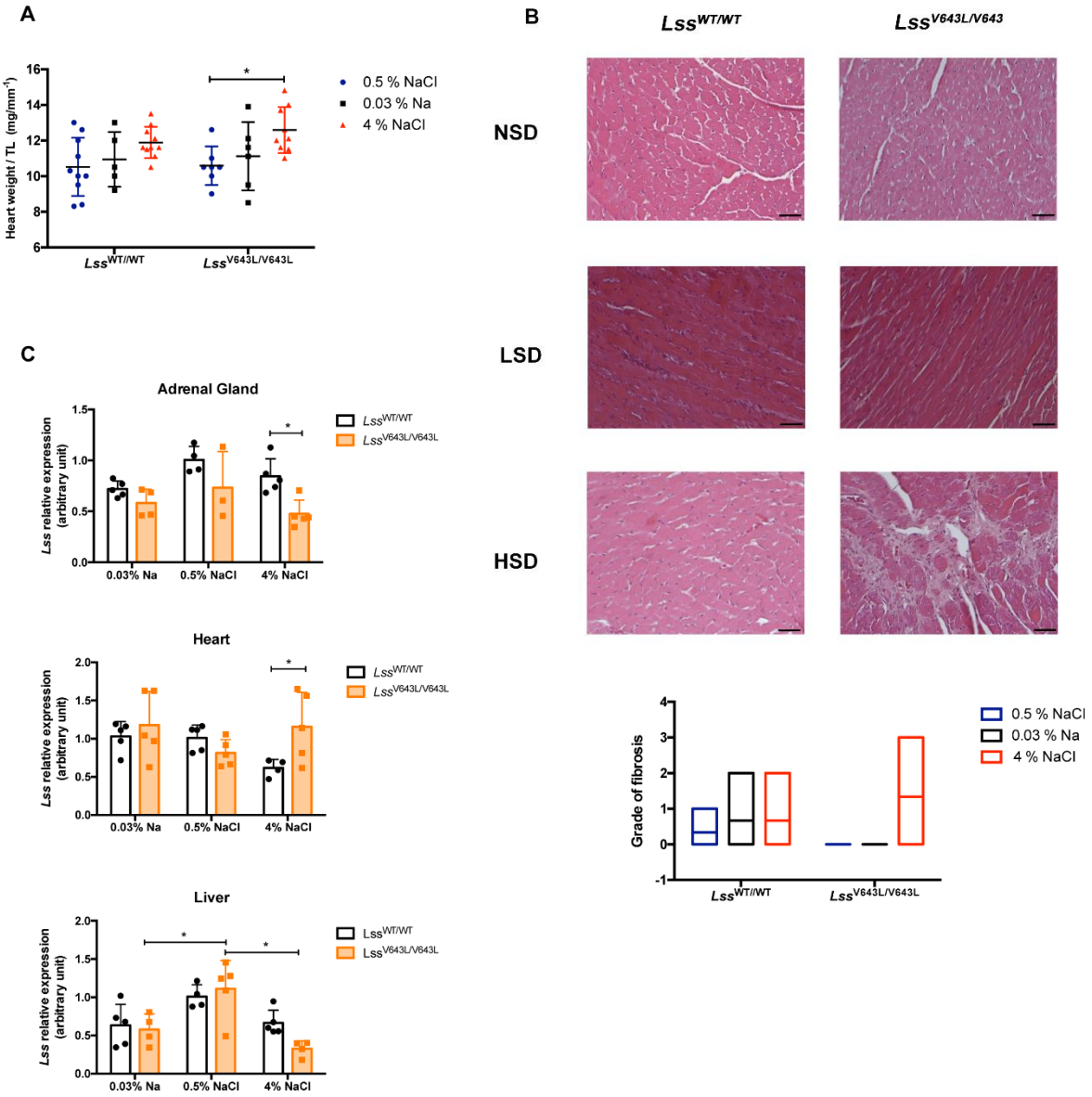


***Figure 14. Hypertension-mediated organ damage and Lss mRNA expression analysis in mice upon NSD, LSD, and HSD diets A.*** *Heart weight and tibia length ratio in* $Lss^{V643L/V643L}$ *and* $Lss^{WT/WT}$ *mice at 12 months of age, under control diet (blue dots), low salt (dark square) and high salt (red triangle) diets (n = 5-10). Statistical significance was assessed by two-way ANOVA (p < 0.05 %).* ***B.*** *Representative histological analysis of heart slices stained with H&E. Bar corresponds to 10 µm. The bar graph represents the quantification of histopathological changes graded on a scale of 1 (minimal) to 5 (marked) (n=3)* ***C.*** *Lss mRNA quantification relative to the Gapdh, in heart, adrenal gland, and liver of 12-month-old mice under NSD, LSD, and HSD. Data are normalized to the average ΔCt values of* $Lss^{WT/WT}$ *NSD mice for each organ (n = 4-5).*

## 3.7 Kidney transcriptome profiling of *Lss*$^{V643L/V643L}$ mice

To deepen the effect of V643L mutation on electrolytes excretion and UFR, the total kidney of mice at 12 months of age, was used for transcriptome profiling by RNA sequencing (RNA-seq).

Principal component analysis (PCA) revealed that the mice's kidneys were not well grouped based on the genotypes and diets (Appx Figure 1).

If we considered the comparison between diets in the same genotype, the quantification and gene types change. Indeed, in *Lss*$^{V643L/V643L}$ mice, the comparison between control and high salt diets revealed that the only differentially expressed gene (DEG) is *Cyb5b*. The same analysis in *Lss*$^{WT/WT}$ analysis reveals 29 DEGs (Figure 15A).

Of 14,988 transcripts, we found only 3 genes (*Fcer1g; Slc8a1; H2-Eb1*) as differentially expressed (FDR<0.05) between *Lss*$^{V643L/V643L}$ and *Lss*$^{WT7WT}$ in the control diet. Instead, in HSD the comparison revealed 10 DEGs, among which *Pllp, Slc10a2*, *Slc29a3*, *Arhgap26*, and *Cbr2* (Figure 15B).

We confirmed some genes (*Fcer1g, Arhgap26, Slc10a2*) by targeted qPCR. Other tested genes were not confirmed and *Slc29a3* showed a different result from RNASeq if analyzed by qPCR (Figure 15C).

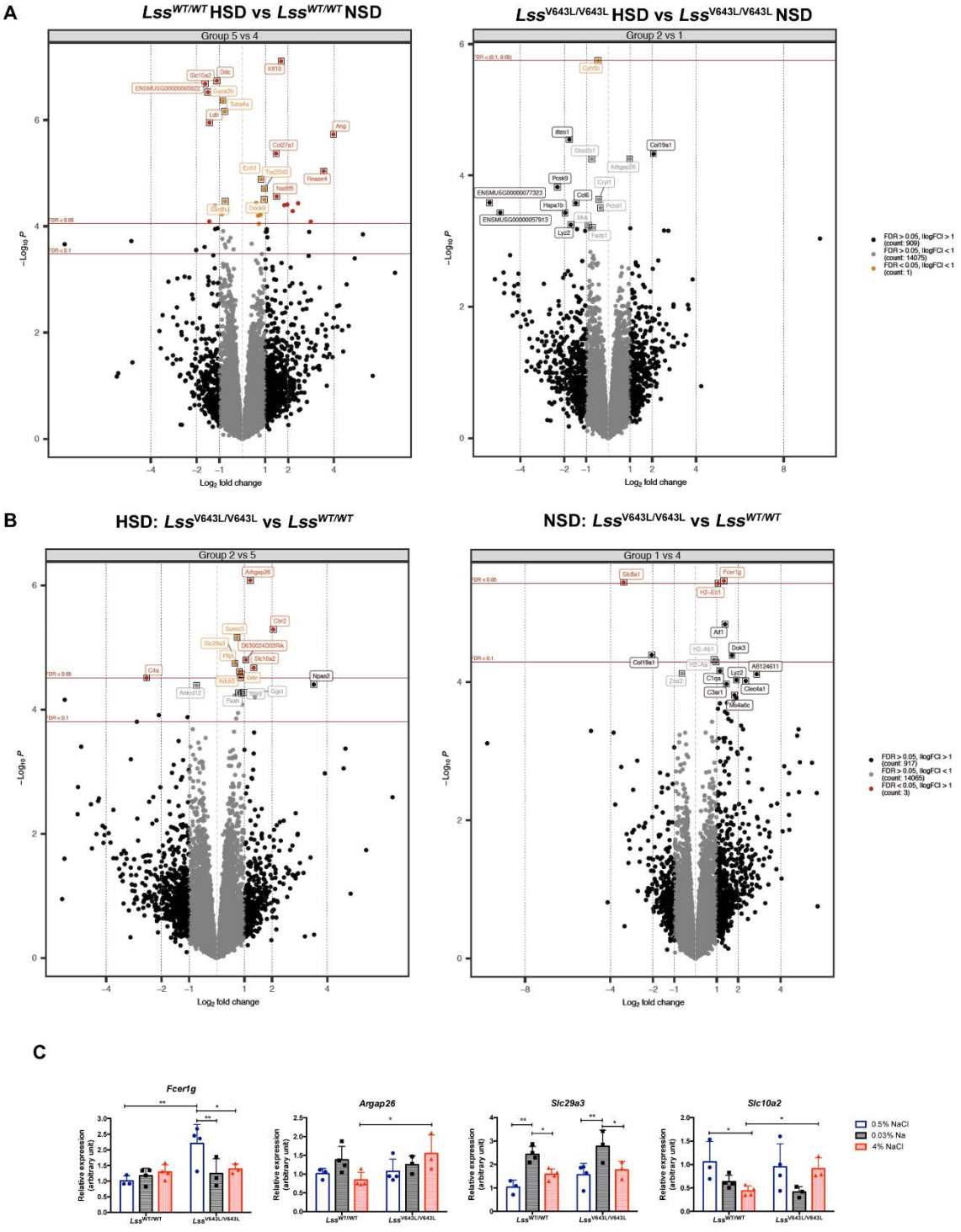


***Figure 15. RNA sequencing analysis of total kidney in Lss$^{WT/WT}$ and Lss$^{V643L/V643l}$ mice under control and high salt diets.*** *Volcano plots representation of DEGs identified by comparison of* ***(A)*** *Lss$^{WT/WT}$ and Lss$^{V643L/V643L}$ mice in HSD vs their relative control mice in NSD, and* ***(B)*** *Lss$^{V643L/V643L}$ compared to Lss$^{WT/WT}$ in HSD and NSD.*

# 4. DISCUSSION

The salt sensitivity of BP is an independent risk factor for hypertension and CVDs. This physiological trait is more frequent in hypertensive patients but was also observed in the general population.

Elevated levels of EO, a cardiotonic steroid, were associated with hypertensive disease and salt sensitivity. In our laboratory, we previously found that the A allele of rs2254524 SNP in the LSS gene correlated with salt-sensitive hypertension, different regulation of EO production, and circulating levels in naïve hypertensive patients. However, the pivotal role of LSS polymorphism in the regulation of EO production and the onset of the disease was not fully elucidated.

This project aimed to dissect the molecular mechanism and the underlined pathophysiological pathways, by which the genetic variant rs2254524 (V642L) of LSS is associated with the development of renal and vascular damage, therefore promoting the onset of hypertension. We took advantage of the CRISPR-Cas9 technology for the generation of a new *Lss* KI mouse to be used as a model for unraveling the *Lss* role in hypertension and steroid biosynthesis.

In this study, we demonstrated that SBP in $Lss^{V643L/V643L}$ mice increase after high salt diet. We also demonstrated that the *Lss* mRNA level of mutant mice is differentially regulated after a high salt diet in different organs. We observed an increased urinary $K^+$ excretion level in mutant mice on a high salt diet. Finally, mutant mice showed increased heart hypertrophy in a high salt diet compared to WT mice.

The mouse generation took more than one year because the first approach failed. Indeed, we first try to microinject CRISPR-Cas9 directly in the zygote, but it had never been given KI mice, therefore, we moved to embryonic stem cell manipulation. Then, we started the project by checking if the new *Lss* KI mouse model confirmed all the findings in humans and its suitability as a novel model for hypertension syndrome.

Initially, we decided to perform the basal characterization in mice at two different ages, 3 and 12 months, first because data collected in humans showed that SBP and DBP level was different in patients carrying the V642L SNP in humans early in life, already in

adolescence (Bigazzi *et al*, 2020), and second because aging correlated with increased salt sensitivity (Kawarazaki *et al*, 2020).

The lifespan in mice and humans is unique. Based on the C57BL/6 survival curve, it was identified three different life phases in mice - mature adult, middle-aged, and old – that have been compared with the human phase (Table 3). In line with this classification, we selected the 12 months of age because is the relative comparison with the age of naïve hypertensive patients (mean 44.9 y) treated with a low salt diet and stratified for LSS genotypes (Lanzani *et al*, 2016).

**Table 3. Mouse life phases with relative ages compared to human age. Data from Flurkey K *et al*, (2007)**

| Mouse life phases | Mouse age | Human age equivalent |
|---|---|---|
| **Mature adult** | 3 – 6 months | 20 – 30 years |
| **Middle-aged** | 10 –14 months | 38 – 47 years |
| **Old** | 18 – 24 months | 56 – 69 years |

No phenotypic differences were observed between the *Lss*$^{WT/WT}$ and *Lss*$^{V643L/V643L}$ mice, indeed the mice were indistinguishable with a similar body weight along age. This result is expected because, in humans, we have never observed differences other than the salt sensitivity of BP and the higher risk to develop AKI after cardiac surgery for patients carrying the A allele (V642L) (Lanzani *et al*, 2016; Iatrino *et al*, 2019). However, anatomical organ evaluation revealed a difference in kidney body mass at 3 and 12 months of age. Besides kidney anatomical differences, *Lss*$^{V643L/V643L}$ mice, both male and female, showed an enlarged liver/body weight ratio compared to *Lss*$^{WT/WT}$ mice. We did not explore the causes of these differences but we can speculate that could be related to lipid accumulation for a possible cholesterol metabolism dysregulation in the liver (Tanaka *et al*, 2013). This finding could be related to the observation that high salt intake in mice is associated with nonalcoholic fatty liver disease (NAFLD), followed by hypertension and cardiac dysfunction in mice (Gao *et al*, 2022).

The *Lss* mRNA quantification in *Lss*$^{WT/WT}$ and *Lss*$^{V643L/V643L}$ mice revealed no differences at both 3- and 12-month time points at the basal level. These data differ from what was observed in human nephrectomies and adrenocortical cell lines transfected with A and C variants; indeed, cortex kidney LSS mRNA analysis has demonstrated a reduction in

mRNA level associated with the A variants of rs2254524 (Lanzani *et al*, 2010). These differences in mRNA levels between mice and humans could be explained by multiple backgrounds or/and different lifestyles in humans that could affect LSS mRNA levels. Interestingly, in humans, another remarkable SNP, rs914247, is in linkage disequilibrium with rs2254524 and is in the 3'UTR of the LSS gene, a position that can affect the mRNA production and influence mRNA analysis in patients. On the contrary, the *Lss* KI mice carried only the rs2254524 genetic variation, and no other differences with *Lss* WT mice that could affect *Lss* mRNA production were present. It would be interesting to introduce this additional and associated SNP in the 3' UTR of the *Lss* gene and deepen its effect on the mRNA levels.

In this study, we did not detect LSS protein levels in mice because the antibody commercially available did not work properly. However, i*n silico* algorithm predict a benign variant for the V642L mutation because it is a conservative change (https://www.ncbi.nlm.nih.gov/clinvar/variation/677175/?new_evidence=true).

Concerning SBP and the rs2254524, Bigazzi *et al*., (2020) demonstrated that this variant was associated with a higher level of SBP and DBP in adolescents carrying the A alleles compared to the one carrying the C. Such data was not observed in *Lss* KI mice, but it is not surprising as the 2638 adolescent participants in the study showed a 1.1 mmHg difference in SBP values. Nevertheless, the mice sample size used in this study was considerably lower to allow any statistically significant difference of such an amount.

If we look at plasma EO in human studies, elevated circulating levels of EO were associated with hypertension (Manunta *et al*, 2001, 2008b). In this study, we have never measured circulating levels of EO in mice for technical reasons. The RIA method used for EO measurements required a large amount of plasma that is difficult to withdraw from mice. Conversely, we performed EO measurements on the total kidney, but mice did not show any statistically significant differences in EO amount differently from what we previously observed in Iatrino *et al*., (2019) demonstrated a higher concentration of EO in both cortex and medulla of AA human nephrectomies compared to CC.

When mice underwent different salt intakes, we demonstrated that $Lss^{V643L/V643L}$ mice shows an increase in SBP after 10 days of 4% NaCl diets (HSD) compared to

*Lss*$^{V643L/V643L}$ mice under a control diet (0.3% NaCl). The genetic background of mice was shown to have a role in the development of hypertension and correlated renal diseases. C57BL/6 mice are considered a salt-resistant strain because it was reported that a high-salt diet is not able alone to increase BP (Lerman *et al*, 2019). Furthermore, another mice model, the 129/Sv, is considered more prone to DOCA-induced hypertension and renal damage compared to the C57B/6 strain, and the backcross of 129/Sv and C57BL/6 mice had an intermediate phenotype (Hartner, 2003). Recently, Ralph *et al*, (2021) demonstrated by radio telemetry technique, considered the golden standard for BP measurements in mice, that C57BL/6 mice are salt-sensitive when subjected to a high-salt diet (3% Na). In our study, we used a C57BL/6 x 129/Sv mixed background, and the $Na^+$ concentration used in the study by Ralph *et al*, (2021) (3%) is double the $Na^+$ concentration of the high-salt diet used in this study (1.5%). This allows us to suppose that the rs2254524 SNP in *Lss* mice is correlated with the salt-sensitive response of BP and that the salt concentration used is not able to develop salt sensitivity in C57BL/6 mice *per se*.

At 12 months of age, we would have expected not only an increase in SBP after high salt diet but also a decrease in SBP in those mice under a low salt diet (Lanzani *et al*, 2016), but this is not the case for *Lss* KI mice. However, another mouse model of salt-sensitive hypertension (AS$^{hi/hi}$ mice) that affects aldosterone synthase showed an increased level of SBP after a high-salt diet but not a decreased SBP if administered a low-salt diet (Makhanova *et al*, 2008).

The volume-loading theory of salt sensitivity argues that the kidney plays a pivotal role in the regulation of SBP upon high salt load due to its role in the regulation of electrolytes excretion. Therefore, we analyzed electrolytes ($Na^+$, $Cl^-$, $K^+$) excretion in urine and, as expected, we observed an increased excretion of $Na^+$ and $Cl^-$ after a high salt diet in both *Lss*$^{WT/WT}$ and *Lss*$^{V643L/V643L}$ mice without any differences between genotypes. In our *Lss* V643L mice, we did not observe $Na^+$ and $Cl^-$ retentions that are commonly used to explain the salt-sensitivity in other models such as the most useful Dahl rat model, Klotho mice or *Umod* mice (Dahl *et al*, 1962; Zhou *et al*, 2015; Trudu *et al*, 2013). However, this theory of salt sensitivity as an alteration of the pressure/natriuresis mechanism is not sufficient to explain such a complex phenomenon. Indeed, Ralph *et al*, (2021) showed

that $Na^+$ excretion *per se* was not impaired and without pressure natriuresis response alteration in salt sensitive C57BL/6 mice. In these mice, the response of BP to a high-salt diet is correlated with sympathetic overactivity. This is also in line with the studies in patients that demonstrated that $Na^+$ excretion in salt-resistant and salt-sensitive patients is quite similar (Cuka *et al*, 2022).

*Lss*$^{V643L/V643L}$ mice showed a reduced urine flow rate, that is the µl of urine excreted per minute per body weight of the mouse. This reduced fluid excretion in mutant mice could cause a volume overload that could be responsible for the hypertensive phenotype.

Another interesting result obtained from this study is the increase in urine $K^+$ excretion in *Lss*$^{V643L/V643L}$ mice in a high salt diet compared to WT mice in the same diet protocol. Surprisingly, this finding is different from the expected one, as we supposed that a reduction in $K^+$ ions excretion must be followed by hypokalemia, which is a low level of $K^+$ in plasma, but this is not the case.

$K^+$ is the most abundant ion in extracellular fluid and more than 77% of $K^+$ intake with the diet is excreted by the kidney. $K^+$ is completely filtered by the glomerulus and most of it is reabsorbed in the proximal tubule and loop of Henle. $K^+$ secretion starts in the distal convoluted tubule and then in the collecting duct and depends on renal outer medullary K (ROMK) (Palmer, 2015).

To investigate possible transporter that could explain the differences in the $K^+$ excretion, we took advantage of data derived from RNA sequencing analysis on the total kidney of *Lss*$^{V643L/V643L}$ and *Lss*$^{WT/WT}$ mice in the three different diet conditions. Unfortunately, this analysis does not reveal any direct differences in the most common transporter that could play a role in $K^+$ homeostasis or their mRNA regulators. However, the comparison between differentially expressed genes identified in *Lss*$^{V643L/V643L}$ and *Lss*$^{WT/WT}$ mice under a high salt diet revealed the upregulation of plasmolipin (Pllp). Plasmolipin is a proteolipid protein first characterized in the brain and kidney (Fischer & Sapirstein, 1994). Plasmolipin inserted in the lipid bilayer triggered the formation of $K^+$ selective channels in vitro (Tosteson & Sapirstein, 1981).

The association between urinary $K^+$ level and CKD, cardiovascular outcomes, and mortality is conflictual. Human studies showed that higher $K^+$ excretion in urine is associated with a low risk of developing hypertension and cardiovascular events (Mente

*et al*, 2014; O'Donnell *et al*, 2014). He *et al*, (2016) demonstrated that patients with CKD with a higher loss of $K^+$ in urine have a higher risk of CKD progression. Conversely, other observational study reported that low urinary $K^+$ level is associated with CKD (Kim *et al*, 2019). Our analysis of kidney functionality and histology did not show any differences between genotypes at 3 and 12 months of age. Renal damage in animal models is different, for example spontaneously hypertensive rats (SHR) developed very slowly kidney damage, in contrast to spontaneously hypertensive rats and stroke-prone rats (SHRSP) under a high salt diet showing a fast development of kidney damage and renal fibrosis that is in line with their higher BP levels (Griffin, 2001; Griffin *et al*, 2014). It is plausible that for renal damage developing or reduced functionality we need a longer high salt diet timing and/or older mice such as 16 months of age. Indeed, Iatrino et al, (2019) demonstrated that AA genotypes were at high risk of reduced eGFR in a hypertensive prospective cohort (median follow-up 4 years, range 1-15 years).

Moreover, RNA sequencing analysis revealed some genes that are differentially expressed in the kidney between mutant mice and WT counterparts in control and high salt diets. The *Slc10a2* is upregulated in mutant mice under a high salt diet. It encodes for the apical sodium-dependent bile acid transporter (ASBT) that is responsible for bile acid transport in the renal proximal tubule, a fundamental mechanism for bile acid conservation. Bile acids are synthesized from cholesterol in the liver.

The *Slc29a3* gene encodes for the equilibration nucleoside transporter (ENT3) that plays a role in the nucleoside uptake in the cell but showed, by real-time PCR, the same pattern of regulation in mutant and WT mice, not consistent with RNA sequencing data. *Arhgap26* is a Rho GTPase activating protein 26 commonly associated with neurodegenerative disorders. Wang *et al*, (2022) demonstrated that several SNPs in this gene were associated with cardiovascular disease. In our study, it was upregulated in mutant mice compared to WT in a high-salt diet.

Fc Epsilon Receptor Ig (*Fcer1g*) was increased in $Lss^{V643L/V643L}$ in the control diet compared to $Lss^{WT/WT}$ mice and it was found associated with atherosclerosis (Huo & Wang, 2021).

We also found a difference between genotypes in the control diet in the *Slc8a1* gene, but this was not confirmed by quantitative PCR. *Slc8a1* encode for NCX1 transporter which is involved in the regulation of peripheral vascular resistance and mediates the EO

regulation of $Na^+/K^+$-pump. Citterio *et al*, (2011) found two SNPs associated with variation of SBP in patients.
These genes rarely or never have been associated with hypertension in patients or with kidney disorders, probably confirming that the salt-sensitive hypertension effect observed in the *Lss* gene is not due to an impairment of kidney function in 12-month-old mice, but these data need to be verified in older mice. Even kidney histological analysis did not reveal any differences between genotypes.

*Lss* mutant mice in a high salt diet showed an interesting increased heart weight/tibia length ratio versus a control diet, with the histological analysis showing an increased level of cardiac fibrosis compared to the WT mice. It is compelling evidence that the most important clinical consequence of hypertension is hypertension-mediated organ damage (HMOD). Our group recently published that salt sensitivity (and inverse salt sensitivity) appear to be equivalent risk factors for CV events compared to salt resistance and the response to an acute saline test is predictive of CV damage in a hypertensive prospective cohort (Cuka *et al*, 2022). In different animal models is demonstrated that elevation in BP causes an increased left ventricular hypertrophy in 2 to 4 weeks of high BP (Crowley *et al*, 2006). This data confirmed this model as a possible mouse model for studying salt-sensitive hypertension.

One of the most interesting findings of our study revealed a different regulation of *Lss* mRNA expression depending on the rs2254524 SNP affected by salt content. Indeed, high-salt diets reduced *Lss* mRNA levels in mutant mice but not in the WT counterparts fed with high-salt diet too. Moreover, we also observed a reduction in the *Lss* mRNA level in the liver of mice, the organ with higher expression of *Lss* transcript.
LSS is an enzyme involved in the pathway of steroids, including cholesterol, but it also plays a key role in alternative oxysterol pathways and catalyzes the formation of 24(S), 25-epoxycholesterol that are activators of liver X receptor (LXR) (Brown, 2009). It was demonstrated that in a condition of partial inhibition of LSS, the oxysterol pathways are favored. Alteration in endogenous LXR activity was correlated with different pathologic alterations including cardiovascular disease.

This study has some limitations. The first is the mixed background of mice that we used to perform experiments. However, after 10 generations of backcross with C57BL6 mice, we are now obtaining the congenic mice necessary to exclude any possible differences due to distinct genetic backgrounds. Second, the current technique used in this project for BP measurements, the tail-cuff system, was not considered the golden standard for such a measurement. Nevertheless, we considered our BP measurements reliable because we assessed the training of the mice by evaluating, during BP measurement, the decreased animal movement in the mouse restrain as the days of measurements increased and the decrease of feces and urine, which are a sign of stress reduction. Still, we could validate our SBP data by radio-telemetry technique. Finally, we did not perform aldosterone measurements during the high and low salt diets.

Based on the results obtained and the limitation identified, we plan to validate *Lss*$^{V643L/V643L}$ mice founding in the congenic mice to exclude any background influence. Moreover, we will quantify lanosterol to investigate if the V643L mutation in the LSS enzyme affects its activity together with measurement of aldosterone, which is also needed to investigate its regulation during salt load in *Lss* mice.
Finally, 12-month-old mice mainly studied in this project are in the middle-aged phase, as naïve hypertensive patients usually studied in our laboratory, but we plan to investigate further organ damages also in aged mice at 16-18 months, particularly at the kidney level.

In conclusion, this study confirmed the association between the rs2254524 polymorphism in the *LSS* gene and salt sensitivity in a newly generated mice model (*Lss* KI), previously observed in a cohort of naïve hypertensive patients. We also demonstrated that the *Lss* mRNA level of mutant mice is differentially regulated after a high salt diet in different organs. Salt sensitivity of BP in mutant mice does not appear to be due to an increase in $Na^+$ retention and therefore in renal impairments in 12-month-old mice, although we observed an increased urinary $K^+$ excretion level in mutant mice on a high salt diet. Finally, mutant mice showed increased heart hypertrophy in a high salt diet compared to WT mice, thus indicating hypertension-mediated organ damage affected by this polymorphism and salt intake.

# 5. MATERIALS AND METHODS

## *Lss* V643L mice generation

*Lss*$^{V643L/V643L}$ knock-in mice were generated in collaboration with the Core Facility for Conditional Mutagenesis (CFCM) at San Raffaele Scientific Institute. Cas9 protein, sgRNA, and donor oligonucleotide (Sigma-Aldrich) were nucleofected in embryonic stem cells F1 from a 129/SvXC57BL6 cross. The sequences are reported in Table 4. The proper mutation insertion was first analyzed by RFLP-PCR analysis by the BsgI enzyme (5'…GTGCAG$(N)_{16}$//…3'). CFCM performed the Surveyor Nuclease Assay to evaluate the single base mismatch of mutated clones. Finally, mutations insertions were verified by the sequencing of the entire exon 20 of genomic DNA (Forward primer: 5'-CAGAGGGTTTTCCTCTGTGC.3'; Reverse primer: 5'-GGCTACCTCCTCAAAGCTGA-3').

The recombinant clones were microinjected into C57Bl/6 morulae and coat color chimeras individuated as the mice carrying the mutation. Chimeric males (80% - 100% chimerism) have been mated to C57BL/6 females to transmit the knock-in allele through the germline and the obtained heterozygous mutated mice were then intercrossed to obtain the *Lss*$^{V643L/V643L}$ knock-in mice. Genotypes were distinguished by ASO-PCR with one common primer (Forward primer: 5'-AGTTAGGGATGTTGCGCTGA-3') paired with a primer specific for the wild-type SNP (Reverse primer: 5'-TGGGACCTAGCACTCTGCA**C**-3'), or a primer specific for the inserted mutation (Reverse primer: 5'- CCTAGCACTCTGCA**A**ATACCGT-3').

All animal procedures were performed on C57BL/6 x 129/Sv mixed background mice (F3-F5 generation) at San Raffaele Scientific Institute, Milan, Italy, as approved by Institutional Animal Care and Use Committee - I.A.C.U.C. San Raffaele (Research project n. 846, Authorization n. 360-2018-PR). Mice were housed in rooms with controlled temperature, humidity, and 12 hours of dark/light cycle, with food and tap water *ad libitum*.

For protocol diets, mice were fed with control diet 0.5 % NaCl (0.2 % Na; E15430-047, SSNIFF), sodium deficient diet (≥0.03 % Na; E15430-247, SSNIFF), or high sodium 4 % NaCl (1.71 % Na; E15431-347, SSNIFF) for 3 weeks.

**Table 4. sgRNA and donor oligonucleotide sequences**

| | **Sequences** |
|---|---|
| **sgRNA** | CTTTGAATCATGTGAACAG |
| **Donor oligonucleotide** | gtcccagcagatggcggatggaggctggggagaggactttgaat catgtgaacagcg**A**cggtat**T**tgcagagtgctaggtcccaggtc catagcacctgctgggccctgatgggtttgat |

## Specimen collection

For urine collection, mice were housed in individual metabolic cages for 16 hours overnight. We used the second collecting urine after 1 nonconsecutive day of training. We collected the blood samples from the retro-orbital plexus after isoflurane 2 % sedation, at the sacrifice the same day of urine collection.

All tissues, collected after the whole animal perfusion with cold PBS (Phosphate Buffered Saline), were weighed and frozen in liquid nitrogen for RNA extraction.

Blood and urine analysis were performed by department di Medicina Veterinaria e Scienze Animali, Università degli Studi di Milano, Milan, Italy.

## Endogenous ouabain measurement

Kidney frozen samples were homogenized, by IKA T10 basic ULTRA-TURRAX, Germany), in 1 ml of methanol and then incubated overnight at 4°C on a rotating wheel. Thereafter, the homogenates were all separated by centrifugation (3000 g/min) for 45 minutes at +4°C and the supernatant was dried by vacuum centrifugation (SpeedVac SAVANT system). Samples were reconstituted in 0.025% trifluoracetic acid (TFA) and passed through C18 columns (Bond Elut LRC, Agilent Technologies, VWR) previously washed two times with 3 ml of acetonitrile 100% and for 3 times with 5 ml of double distilled water (dd$H_2O$); then each column was respectively washed 3 times with distilled water and one wash with 3 ml of acetonitrile 2.5%. Following the washing phase, EO was eluted from each column with 4 ml of acetonitrile 25% and then dried under vacuum in SpeedVac SAVANT system.

Samples were resuspended in 110 µl of RIA buffer solution (BSA 1%, $Na_3PO_4$ 0.5M, Thimerosal 0,1%, pH7.4) and EO was measured by RIA-SPA method. Each sample was incubated with [3H] ouabain (30 to 40 Ci/mmol, Perkin Elmer; 2 nmol/L final

concentration), anti-ouabain antibody (custom by DBA, 1:2000 in RIA buffer), and secondary antibody (goat anti-rabbit) conjugated with SPA beads (RPN140 Ysi SPA beads by Perkin Elmer). The plate was incubated overnight at 4°C and then counted on a beta counter (1450 Microbeta, Perkin Elmer).
EO levels were calculated through the Kaleidagraph software. The intra- and inter-assay coefficient of variation was approximately 7% to 10%.

## Blood pressure measurements

The SBP and heart rate were measured by the tail-cuff system (BP-2000 Blood Pressure Analysis SystemTM, Visitech Systems), in conscious mice every 2 days for 10 days to avoid stress. We perform 5 preliminary measurements and 10 actual measurements (Figure 11A) per mouse from 2 to 5 pm. The mouse was placed on a warm platform in a quiet room for the measurements.
We averaged all daily actual measurements for the analysis and considered only mice that have at least 5 actual measurements per day. We excluded the mean with a standard deviation greater than 10.

## Quantitative real-time PCR

Total RNA from tissues was isolated with RNeasy Mini Kit (Qiagen, Cat. 74106). We checked the RNA quantity and purity with Nanodrop 2000 (Thermo Scientific), and the RNA integrity by 1 % Agarose gel electrophoresis. To avoid genomic DNA contamination, 1 μg of RNA was purified with DNase I, Amplification Grade (Cat. 18068015, Invitrogen) before reverse transcription by High-Capacity cDNA Reverse Transcription Kit (Cat. 4368814, Applied Biosystems). Genes were amplified by qRT-PCR with SYBR Green PCR Master Mix (Cat. A25742, Applied Biosystems). Primer's pair used were reported in the table below.

| **Genes** | **Forward primer (5'-3')** | **Reverse primer (5'-3')** |
|---|---|---|
| *Gapdh* | TGCACCACCAACTGCTTAGC | GGATGCAGGGATGATGTTCT |
| *Lss* | GCTTGTTCGTTGGTCAGTGG | ATCCATTGGTGCCCTGCATT |
| *Fcerg1g* | AAGATCCAGGTCCGAAAGGC | TCTGAAGCTACTGGGGTGGT |

| | | |
|---|---|---|
| *Arhgap26* | AGAGACGCTCAAGTCACACG | CCGCTTTGCCGAAGACAAAT |
| *Cbr2* | CATGGGCAAGAAAGTCTCTGCAG | ACTGGTAGAGGCACTTCTGTCG |
| *Cyb5b* | AAGCGAAACTCGGCGGAAGAGA | TGTTCCAGCAGAACCTCTTCCC |
| *Slc29a3* | CCTGGAACTTCTTTGTCACTGCT | GCAACTGCCAGGTAGCTCTCAA |
| *Slc10a2* | GGTTTCTTCCTGGCTAGACTAGC | GGAAGGTGAACACCAGGTTGAG |
| *Slc8a1* | AGCCCTGTTGTTGAATGAGCTTGGT | CCAGCTTGGTGTGCTCGCCT |

Gene expression was quantified with the $2^{-\Delta\Delta Ct}$ method. *Gapdh* reference gene was used for the normalization of mRNA expression. The Mean ΔCt of *Lss*$^{V643L/V643L}$ mice was expressed relative to Lss$^{WT/WT}$ mice in the control diet (ΔΔCt) and shown as 2-ΔΔCt fold changes.

We analyzed the samples in triplicates for *Lss* mRNA quantification and duplicates to confirm RNA sequencing analysis.

## Histological staining

The kidney and heart were fixed in 4% paraformaldehyde for histological evaluation. and embedded in paraffin. 5µm sections were stained with hematoxylin and eosin (H&E) to evaluate the tissue morphology. Histopathological changes were graded on a scale of 1 to 5 as minimal (1) to marked (5).

## RNA Seq

RNA from the total kidney was extracted as described above. The RNA sequencing and bioinformatic analysis were performed by LIFE & BRAIN Genomics (Bonn, Germany). mRNA library was made by QuantSeq 3' mRNA-Seq Library Prep kit fwd (Lexogen). RNA-prepared sequencing was performed by the NovaSeq 6000 sequencing system (Illumina).

## Statistical analysis

Statistical analysis was performed with GraphPad-Prism software. Comparisons between 2 groups were performed by two-tailed unpaired Student's t-test.

We performed multiple comparisons by two-way ANOVA test followed by Tukey's multiple comparisons.

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Sportine Faienza

# 7. APPENDIX

**Appx Table 3. Basal characterization of female KI mice at 3 and 12 months of age**

| | 3 months | | 12 months | | *p-value* |
|---|---|---|---|---|---|
| | **$Lss^{WT/WT}$** | **$Lss^{V643L/V643L}$** | **$Lss^{WT/WT}$** | **$Lss^{V643L/V643L}$** | |
| Body weight (g) | 24. 49 ± 4.1 | 22.61 ± 2.6 | 38.80 ± 7.5 | 35.56 ± 6.1 | |
| SBP (mmmHg) | 106.7 ± 4.6 | 102.2 ± 4.4 | 101.34 ± 1.9 | 103.4 ± 5.7 | |
| Kidney/BW (mg/g) | 8.7 ± 1.3 | 7.5 ± 0.6 | 4.83 ± 0.9 | 5.6 ± 1.0 | |
| Liver/BW (mg/g) | 54.3 ± 4.4 | 50.7 ± 4.4 | 32.9 ± 5.2 | 44.5 ± 4.3 | * |
| Heart/BW (mg/g) | 5.9 ± 1.5 | 5.9 ± 0.5 | 4.12 ± 0.9 | 4.9 ± 0.8 | |
| Brain/BW (mg/g) | 20.1 ± 3.8 | 21.4 ± 2.0 | 14.2 ± 3.3 | 15.02 ± 1.8 | |

Data are mean ± SD; *n* = 5

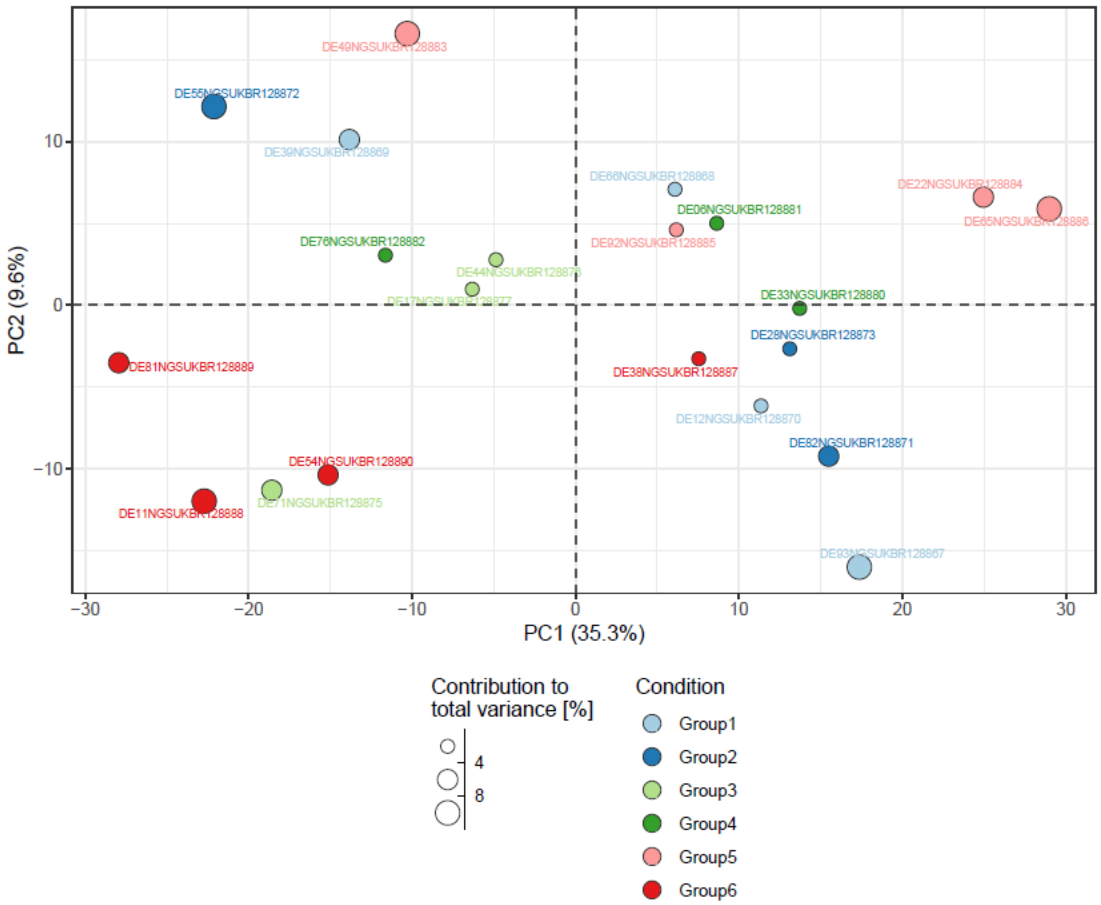
SBP: Systolic Blood Pressure

* $Lss^{WT/WT}$ vs $Lss^{WT/WT}$ at 12 months of age (*p<0.05)*

**Appx Table 4. Biochemical Parameters of female KI mice at 3 months of age**

| | **$Lss^{WT/WT}$** | **$Lss^{V643L/V643L}$** |
|---|---|---|
| **Serum** | | |
| Creatinine (mg/dL) | 0.38 ± 0.02 | 0.38 ± 0.07 |
| Cholesterol (mg/dL) | 60.00 ± 9.54 | 63.66 ± 4.07 |
| Urea | 54.4 ± 10.15 | 49.17 ± 8.61 |
| **Urine** | | |
| Diuresis (µl) | 1.06 ± 1.03 | 0.52 ± 0.35 |
| Urine Flow Rate (µl/min/g) | 0.05 ± 0.05 | 0.02 ± 0.01 |
| Creatinine (mg/dL) | 44.23 ± 12.3 | 31.58 ± 10.0 |
| $Na^+$/Crea (mM/mg) | 0.47 ±0,06 | 0.52 ± 0.04 |
| $Cl^-$ /Crea (mM/mg) | 0.68 ± 0.10 | 0.65 ± 0.09 |
| $K^+$ /Crea (mM/mg) | 1.05 ± 0.09 | 0.84 ± 0.08 |
| Creatinine Clearance (µl/min/g) | 2.61 ± 0.75 | 2.24 ± 1.38 |

Data are mean ± SD; *n* = 5




**Appx Figure 2. Principal component analysis of RNA sequencing analysis.**