

Investigating the influence of *NEDD4L* in the development of salt sensitive hypertension with age

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ABSTRACT

Background: Hypertension, a leading risk factor for cardiovascular disease, exhibited in 17.7% of the Canadian population, and attributed to 13% of world mortality, is influenced by both the environment and genetics. Salt sensitivity is described at higher rates in the hypertensive population. The NEDD4-like (NEDD4L) protein is important in sodium reabsorption and has been implicated in essential hypertension and salt sensitivity.

Objectives: Two variations (rs4149601/rs2288774) found in NEDD4L have been associated with salt sensitivity and hypertension; a third (rs576416) is in linkage disequilibrium with rs4149601. The purpose of this study is to assess the relationship between the NEDD4L rs4149601, rs2288774, and rs576416 single nucleotide polymorphisms with sodium and age on blood pressure (BP).

Methods: Canadian hypertensive patients were recruited through the University of Ottawa Heart Institute, with genotyped data from Leuven, Belgium, and the DNA of subjects from Warsaw, Poland also included in the study. Eligible subjects were studied off anti-hypertensive medications. Daytime BP was measured using 24hr ambulatory BP monitoring in 662 Caucasian hypertensives (BP \geq 130/85 mmHg). 24hr urine Na⁺ was collected. DNA from Canada and Poland was genotyped on the GeneTitan Affymetrix Axiom platform and through TaqMan MGB probe-based RT-PCR, while the Belgium samples were analyzed on Illumina 1M-duo arrays. Simple and multivariate linear regression modelling with SAS 9.4.0 was used for genotypic comparisons affecting BP, combined with age and corrected urine sodium.

Results: The three hypertensive populations were significantly different ($P < 0.05$) across all demographic and clinical measures, even when stratified by sex. The Polish and female hypertensives from Canada and Belgium were removed from the analysis for lacking the general populations' trend of increasing BP with age. Multiple linear regressive modelling found a significant association ($P_{\text{model}} = 0.0034$) of rs4149601 GA ($P = 0.0129$) and GG ($P = 0.0082$), with age and urine sodium, on SBP in the Belgium male hypertensives ($n = 273$). No significant models analyzing the association of rs576416 and rs2288774 with BP in the Belgium population were found. In the Canadian hypertensive population ($n = 120$) no association on the discrete analyses of the rs4149601, rs576416, and rs2288774 genotypes were found; however the combination of the GG rs4149601 and AA rs576416 ($\beta = 0.021$, $P = 0.03$) and the GG rs4149601 and CC rs2288774 ($\beta = 0.020$, $P = 0.04$) genotypes showed significant associations with BP in borderline significant models ($P = 0.055$ and $P = 0.094$ respectively), when analyzed with urine sodium levels and age.

Conclusions: A significant influence of the rs4149601 G-allele, with urine sodium and age, was found to be associated with an increase in BP in the Belgium males. Multiple linear modelling describing borderline significant findings in the interaction of rs4149601 with rs576416, and rs4149601 with rs2288774 in Canadian male hypertensives suggests of the possible synergism between polymorphisms and development of salt sensitive hypertension. Future research could evaluate the role of NEDD4L on the sex differences in early-onset salt-sensitive hypertension.

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3.0 INTRODUCTION

3.1 Blood Pressure Regulation

Blood is the essence of life, vital for supporting the physiological functions of the human body. It is the major transportation artery for the delivery of nutrients and gases, distribution of hormones, and the removal of physiological wastes. In order to maintain this uninterrupted perfusion of blood to bodily tissues, blood pressure (BP) must be continuously regulated (Lifton, Gharavi & Geller, 2001). It is a complex series of interactions between the environment, genetic make-up, and different physiological systems that influence BP. Physiologically, collections of vasoconstrictors and vasodilators, adrenergic receptors, as well as a multitude of components from the renin-angiotensin-aldosterone system have been documented to impart short-term changes on BP. However, long-term associations of these systems on BP have been difficult to identify because of the complex integration of these systems and physical characteristics – such as age, sex, and body size. Additionally, genetic variations that impart small to moderate effects in protein conformation can further complicate the issue (Lifton, Gharavi & Geller, 2001; Verrey, Hummler, Schild, & Rossier, 2008).

As mentioned previously, BP is a rigorously monitored balance of vasculature, water and salts. The renin-angiotensin-aldosterone system (RAAS) helps stabilize this process through multiple organs, where it signals, using a hormone cascade, the kidneys to regulate water through the retention or secretion of salts. Traditionally, a

major role of RAAS in response to a lowering of BP is to retain water and sodium (Na⁺). Angiotensinogen is produced by the liver and circulates in the plasma. The juxtaglomerular cells of the kidney, in response to a decrease BP or sodium levels, release renin, a glycoproteolytic enzyme, that cleaves angiotensinogen into angiotensin I (AngI). AngI is a biologically inactive intermediate that circulates until it gets converted into the potent peptide hormone angiotensin II (AngII) by the angiotensin-converting enzyme (ACE). ACE is predominately found in the lungs, with some activity in vascular endothelial cells, and epithelial kidney cells, crucial in producing AngII from AngI (Le, Crowley, Gurley, & Coffman, 2008). In addition to converting AngI into AngII, ACE participates in the breakdown of the vasodilator bradykinin. AngII is a dynamic component of the RAAS, signaling not only vasoconstriction but also sodium reabsorption through stimulating the release of aldosterone both directly and indirectly via the pituitary gland.

Aldosterone is a steroid hormone manufactured in the *zona glomerulosa* of the adrenal cortex of the adrenal gland, located immediately superior to the kidneys. Aldosterone is important because it has a strong influence on sodium reabsorption in epithelial cells – classically in the kidneys – though, more recent research has also suggested its influence in cerebral regions could also affect BP (Van Huysse, et al. 2012; Hamlyn, et al. 2014). At the cellular level, aldosterone, an important hormone of the RAAS, binds to the mineralocorticoid receptor (MR) in the cytoplasm to form MR dimers (Figure 1). These MR dimers then translocate to the nucleus and induce transcription of several genes involved in salt, and water exchange. The epithelial sodium channel (ENaC) is a sodium specific channel in the apical membrane of

epithelial cells, which allows sodium ions to cross from the lumen into the cell. Na⁺/K⁺ ATPase, an electrogenic enzymatic pump that exchanges two potassium ions for three sodium ions at the cost of ATP at the basolateral surface of epithelial cells. NEDD4L, from the Need4 family of ubiquitin ligases, that regulates ENaC expression. The serum-and-glucocorticoid-regulated kinase 1 (SGK-1) is a protein kinase that modulates ENaC both directly by stimulating ENaC function, and indirectly by inhibiting NEDD4L through phosphorylation. Aldosterone also induces transcription of 11-β hydroxysteroid dehydrogenase type II (11-βHSD2), a corticosteroid and a competitive inhibitor of MR, and negative feedback mechanism, that reduces the binding affinity of aldosterone to MR. (Lifton, Gharavi, & Geller, 2001; Kusche-Vihrog & Katja Sobczak, Bangel, Wilhelmi, Nechyporuk-Zloy, Schwab, Schillers, & Oberleithner, 2007; Verrey, Hummley, Schild, and Rossier, 2008; Grossmann & Gekle, 2009; Soundararajan, Pearce, & Ziera, 2011). This extensive influence of aldosterone at the molecular level adds to the rigorous and complex nature of BP and sodium regulation.

Under normal conditions the kidney reabsorbs 95-98% of filtered sodium – 70% occurring in the proximal tubule and thick ascending limb. The remaining sodium is reabsorbed in the distal convoluted tubule (DCT) and aldosterone-sensitive distal nephron (ASDN). Even though sodium reabsorption in the DCT/ASDN represents a smaller fraction of the filtered load, it is considered as the important region for renal salt reabsorption with regards to BP (Lifton, Gharavi, & Geller, 2001; Schild, 2004). Salt reabsorption in the DCT and ASDN is partially influenced by the hormones vasopressin and aldosterone, via the amiloride-

sensitive epithelial sodium channel (ENaC) (Verrey et al., 2001; Hawk et al., 1996). The transition of sodium from the lumen is a two step process where ENaC first transports sodium into the cell, while Na⁺/K⁺ ATPase exchanges sodium for potassium ionic molecules at the basolateral surface (Figure1) (Loffing et al., 2001; Verrey, Hummley, Schild, and Rossier, 2008). These components – Aldosterone, MR, Na⁺/K⁺ ATPase and ENaC – are all found in several cerebral tissues: the choroid plexus, ventricular ependymal, and neuronal nuclei. When activated, these components influence the concentration of sodium in the cerebrospinal fluid, which has shown to affect both heart rate (HR) and BP (Huang, et al. 2013), which is discussed further in section 1.3.

Before plunging deeper into the nuanced and intricate nature of BP regulation, it is imperative to highlight views on the causes of BP changes. Lifton, Gharavi, & Geller (2001) provide an excellent overview of the causes of raised BP, from the previously mentioned systems, to the competing causal perspectives – genetics versus the environment – through the analysis of adoption and twin studies. Environmental, or external pressures are purported as having a strong influence on the development of BP changes; as dietary (Appel et al., 1997; Harrington et al., 2013) and physical activity (Cornelissen & Smart, 2013) practices continually show their effects on an individual's BP. Familial studies support this assertion, as they have shown that siblings in the same household have a stronger BP correlation, both systolic and diastolic, than those siblings who live apart, regardless of the number of years away from the home (Longini et al., 1984). This would suggest that a common

environment, the same household, or rather, environmental pressures has an influence on BP. In contrast, twin and adoption studies indicate that genetics also play a pivotal role in determining BP. It was shown that the relationship between BP measures was strongest in monozygotic twins than their dizygotic counterparts (Feinleib et al., 1977). While an adoption study (Biron, Mongeau, & Bertrand, 1976) described natural siblings and their parents, in the same household, had a small, yet significant correlation between BP measurements, unlike the adopted individuals. These studies demonstrate that genetics has an increased importance in the development of hypertension than previously assumed. Rare Mendelian forms of single gene effects on BP – Pseudohypoaldosteronism and Liddle’s Syndrome – strengthen this assertion, and they will be discussed at a later time.

The elucidation of genetic control over BP has been arduous. Investigating the specific components and how they contribute in long-term changes in BP has proven to be difficult, given the complex nature of genetics and hypertension. Variations in phenotype can be observed due to heterozygotic genotypes, which can potentially disguise the effects of a particular gene of interest and its level of penetrance (Jeunernaitre, et al., 1992; Cordell & Clayton, 2005). While some genes are found commonly to impart only small to moderate changes in protein conformation, or involve multiple gene regions that can result in outcomes for complex disorders that are difficult to detect (Cordell & Clayton, 2005). Aside from determining gene versus environmental implications, BP regulation is a dynamic, intricate process with many variables that are a challenge to isolate. Not only are

several variables related and isolating any single variable could negate the effects of another, but the characteristics of some genetic variables could be confounded by the effects of other genetic regions (Cordell & Clayton, 2005; Munroe, Barnes, & Caulfield, 2013). It is understandable that studying the components that moderate BP can be demanding. The following chapters will convey the importance of investigating and identifying the mechanisms that influence the long-term development of BP.

3.2 Hypertension

Hypertension (HTN) is defined by having sustained levels of elevated BP. Classically, normal BP is considered 120 mmHg of SBP and 80 mmHg of DBP, while HTN is commonly characterized by individual BP measures above 140/90 mmHg (Basu & Millett, 2013; Carson et al., 2013). However, several studies have used BP measures between 130-220 mmHg (SBP) and 85-120 mmHg (DBP) to describe the hypertensive phenotype through multiple measurement techniques – such as, averaging three individual point BP measurements (Gu et al., 2013; Niiranen et al., 2014; Staessen et al., 2011). The WHO (2012) describes raised BP as the leading behavioural and physiological risk factor of mortality, attributing HTN to 13 % of global deaths. The prevalence of hypertension is quite astounding, with more than 17.7% of Canadian's (Stats Canada, 2014), and 21% of Ontarian's (Leenen, McInnis & Fodor, 2010) displaying this phenotypic risk factor. More specifically, 14.6 % of the current Canadian Caucasian population has been diagnosed with high BP (Liu, et al., 2010). This is likely an underrepresentation of the actual impact of HTN since it is widely regarded as a symptomless cardiovascular risk factor, labeled the “Silent

Killer” in written work by R.S. Rapport in 1999. With cardiovascular disease being considered a primary global affliction, and HTN as the principal risk factor in its development (Vasan et al., 2002), the investigation into the progressions of HTN becomes paramount.

The development of HTN is considered multifactorial, having both environmental and genetic (Bubien, 2012; Araki, et al., 2008) influences contributing to its etiology. Epidemiological studies have helped identify a collection of factors that influence HTN, including age (Vasan et al., 2002), sex (Maranon & Reckelhoff, 2013), body mass index (BMI) (Gelber, Gaziano, Manson, Buring, & Sesso, 2007a), as well as dietary sodium (Bray et al., 2004), and potassium (Whelton et al., 1997). Age is consistently considered a risk factor for the development of HTN. The elderly portion of the population displays the largest prevalence and some of the greatest incidence rates with regards to HTN (Lloyd-Jones, Evans, & Levy, 2005). A prospective cohort study, the Framingham Heart Study (Vasan et al., 2002), has also shown that the risk of developing HTN increases with the progression of age. This trend is also well reflected in the Canadian population (Figure 2). Nearly 10% of the population below the age of 45 years are found to have HTN, while those 65 years and older represent 47.4% of those with HTN; a large disproportion (Stats Canada, 2014).

Sex differences are also commonly described for HTN, as men and women exhibit increasing BP characteristics at different times. Men have been shown to develop high BP at a much earlier age than their female counterparts (Yanes &

Reckelhoff, 2011; Maranon & Reckelhoff, 2013). Women are much more likely to develop HTN in later life stages, after the onset of menopause - on average 51.4 years of age (Gold et al., 2001) - than men. Post-menopausal women then match, or surpass males when it comes to developing HTN (Yang & Reckelhoff, 2011; Maranon & Reckelhoff, 2013). This trend is once again reflected well in the general Canadian population (Figure 2). Overall, males have higher rates of HTN than females: 18.5% to 17.0% respectively in 2014. The HTN rates found in males are higher in every age category below the age of 65 years, where female HTN rates jump to 48.8%, compared to 45.7% in males (Stats Canada, 2014). Estrogen has been suggested as a protective element for women, as it is largely considered the key component in managing BP during pregnancy, and the balance between androgens and estrogen tends to favour androgens in older females. It has been suggested that the estrogen receptor is implicated in BP regulation by maintaining endothelial vascular tone and can increase the bioavailability of nitrous oxide, a potent vasodilator (Barton & Meyer, 2009). Several pathways on the impact of estrogen on the RAAS have further been hypothesized; however, estrogen hormone replacement therapies have proven to be inconclusive when assessing post-menopausal BP and cardiac outcomes (Reckelhoff & Fortepiani, 2004). A well-established explanation of why younger women are protected remains elusive (Yang & Reckelhoff, 2011; Lima, Wofford, & Reckelhoff, 2012).

Though, the mechanisms are not well-known, increased body weight and body size ratios are associated with increased risks in developing HTN. Several population studies – a large prospective cohort study, and the Physicians Health

Study – have reported that the prevalence of the HTN status to be much higher among groups with a larger measure of BMI in each of the study populations (Thomas et al., 2005; Gelber, et al., 2007a; Gelber, et al., 2007b; Leenen, McInnis, Fodor, 2010). Hypertrophic adipose tissues have been shown to secrete leptin and angiotensinogen. Angiotensinogen, as previously mentioned (section 3.1), is a key component of the RAAS that regulates BP. Meanwhile leptin can activate the sympathetic nervous system (SNS). SNS activation increases norepinephrine turnover and increases its plasma concentration, which in turn can activate the release of renin from the kidneys leading to sodium retention (Ruano et al., 2005). Furthermore, it has been suggested that free-fatty acids, due to high caloric intake of fats and carbohydrates, can further involve the SNS through stimulation of the alpha1 and beta adrenergic receptors leading to salt reabsorption and increasing overall blood volume. (Kotsis et al., 2010). Though these SNS aldosterone releasing factors have been shown to mimic hyperaldosteronism (Fujita, 2010), the results are usually described in subjects with metabolic syndrome, and/or morbid obesity. These purported mechanisms effects on BP and whether they are causal, however interesting, still remains unclear for the general hypertensive population.

Further, another epidemiologic factor relevant to the nature of HTN is potassium. Cardiovascular events are reported at much lower frequencies in populations that consume diets that are considered more primitive (lacking grains and processed foods) and are rich in potassium. It has been proposed that potassium can be effective in stimulating vasodilation via hyperpolarizing endothelial cells that signal vascular smooth-muscle cells to relax (Houston, 2011).

Potassium has been the subject of numerous randomized controlled trials (RCTs) in humans. A meta-analysis completed by Whelton et al. (1997) on 33 RCTs concluded that oral potassium supplementation (60-120 mmol/d) significantly reduced both SBP (-3.11mmHg) and DBP (-1.97 mmHg). More recently, however, a meta-analysis published by the Cochrane Collaboration, that included 8 RCTs, found a strong but insignificant inverse relationship between BP and potassium supplementation (Dickinson et al., 2006). The function and the effectiveness of potassium in lowering BP remains inconclusive, though, it seems that potassium does potentially have a role, albeit minor, in the regulation of BP.

It has been well established that sodium is a critical component in the regulation of BP; with its importance in the RAAS highlighted in section 1.1. Dietary reductions of sodium have been suggested as a way to alleviate the pathogenesis of high BP at the population level (Appel et al., 2013). A dietary trial, comparing two dietary intervention groups – a DASH diet and a typical North American control diet – where subjects from each dietary group were exposed to three 30-day periods with varying levels of sodium in random order, Each group showed a significant reduction in mean SBP and DBP when comparing the low and high salt dietary periods (Bray et al., 2004). Additionally, a meta-analysis of over 50 sodium reduced dietary interventions has shown that a marginal decrease in urinary sodium was paired with significantly lower BP in both hypertensive and non-hypertensive individuals (He & MacGregor, 2002). The studies included improved on many of the drawbacks and flaws inherent in previous works – dietary recall bias – that lead to dietary sodium inaccuracies. The dietary recall issues are overcome through urinary

sodium analysis to estimate dietary sodium intake (Huang, et al., 2014).

Furthermore, urinary sodium can be corrected for creatinine, and is considered an even more stringent measure of sodium intake than the simple urinary sodium concentration analysis. Creatinine is a natural by-product of muscle metabolism, the breakdown of creatine and creatine phosphate, and it is released at a constant rate from the body. Thus, creatinine is commonly used to normalize the concentrations of urine analyte excreted from the body (Barr et al., 2005), such as sodium, as subjects can have varying levels of urine production. This correction improves, or rather standardizes the more accurate 24 hr urinary sodium collection used to estimate dietary sodium.

3.3 Salt Sensitive Hypertension

As we continually mention, BP is a highly regulated process, and salts and water balance play pivotal roles in its homeostasis. Sodium is one of the salts that have widely been revered to have an important effect on BP. Though, the impact of dietary sodium on BP varies within a population. The salt-sensitive phenotype (SS) – salt-sensitivity is defined as “the individual blood pressure reaction to a given change in salt intake” (Dahlberg, Nilsson, von Wowern & Melander, 2007) – is reported at higher levels in hypertensive populations versus their normotensive counterparts. Wright, et al. (2003) describes SS at rates of 64% and 52.1% in hypertensive African American and Caucasian women (age 58.4 +/- 7) respectively. While several investigative groups suggest SS rates above 50% in the hypertensive and between 10% and 26% in the normotensive populations (Weinberger, Miller, Luft, Grim & Fineberg, 1986; Tesson & Leenen, 2007). However, there is no

consensus on how salt-sensitive HTN (SSH) should be clinically diagnosed. The current 'best-practice' involves an extensive, and expensive procedure that requires a strict low-versus-high dietary salt intake protocol. BP measurements on the low-salt are compared to BP measurements on the high-salt diet. A SS classification is assigned if there is difference of ≥ 5 mmHg in either, or both, systolic and diastolic BP between the dietary salt extremes (Franco & Oparil, 2006; Overlack et al., 1993). Furthermore, the SS phenotype has also long been proposed to be predominately controlled genetically, with heritability as high as 74% (Svetkey, McKeown & Wilson, 1996). The prominence of SS in hypertensive populations and the challenges of determining this phenotype, through classic clinical dietary methods, warrant an investigation into the identification of genotypes conferring to the SS phenotype. The proper genetic identification of SSH could help provide some insight to the molecular mechanisms of SS and potentially be a cost-effective diagnostic tool for the prevention and/or treatment of HTN.

Abnormal functioning kidneys, and an inability to secrete high salt loads, are widely regarded as the culprit for SSH. Lower levels of both renin activity and aldosterone concentrations in the plasma are commonly reported in many studies involving SS subjects (Weinberger, 1996). It could suggest abnormalities in the RAAS functioning's, though it was not clear as these eight studies reviewed by Weinberger (1996) did not identify a clear source for the reported reductions. Although, while also unclear, several studies have suggested that physical renal abnormalities could account for sodium retention in SS individuals. Indirect evidence from human and

animal studies has provided evidence that a lower nephron count and reduced glomerular surface area could explain the presentation of this SS phenotype (Weinberger, 1996).

More recently, however, the mechanism determining SS has been challenged as research has shown that brain components may be prominent in determining the SS status. Research on Dahl salt sensitive (DS) and Dahl salt resistant (DR) rats by Morgan, DiBona, & Mark (1990) was an early suggestion for an alternative, non-kidney explanation for the SS phenotype. BP of DS rats' is highly susceptible to dietary sodium intake, where the BP of DR rats is resistive to sodium. This study found that when the DS rats were transplanted with DR kidneys that they still developed HTN on a high sodium diet, suggesting an external factor other than the kidney for determining SS. Huang, Amin, & Leenen (2006) expanded on this preposition to postulate that the mechanism responsible for the SS phenotype may lie in the central nervous system (CNS). It has been highlighted that the aldosterone-MR-ENaC interaction, described in section 1.1, for BP regulation has also been identified in the brain, with high MR levels found in the choroid plexus, ventricular ependymal and several neuronal nuclei. In previous work (Huang, Van Vliet, & Leenen, 2004), they demonstrated that increases of sodium concentrations in the cerebrospinal fluid (CSF) of DS rats on a high salt diet, but found no significant change in the sodium concentration in the CSF of DR rats. This was important as the increase of sodium concentrations in the CSF preceded the observed increases in heart rate and BP. Additionally, an interacerebroventricular injection of artificial CSF, containing high concentrations of sodium, in DR rats can result in a BP

response observed in DS rats on a high salt diet (Huang, Veerasingham, & Leenen, 1998). The issue of SS HTN potentially may not solely reside with the nephrons. Ultimately, the previous studies emphasize the importance of the aldosterone-MR-ENaC interaction in the SS phenotype, whether it lie in the brain or the kidneys.

3.4 Epithelial Sodium (Na⁺) Channel

ENaC is a hetero-multimeric transmembrane sodium transporter expressed in multiple body tissues including the brain, lungs and kidneys. It is an end product of the RAAS as aldosterone signals ENaC transcription when binding to the MR in the cytoplasm of endothelial cells (Figure 1). When active within the kidney, ENaC is located on the apical side of epithelial cells in the DCT and ASDN (Garrone et al., 2009), and it is comprised of three subunits – α , β and γ – that together form a sodium specific passageway critical for salt reabsorption and water balance (Knight et al., 2006; Schild, 2004). Functionally, this protein channel has been targeted in treatment of hypertension. Amiloride and Triamterene are pharmaceuticals that are used in combination with other anti-hypertensives to block ENaC function. Genetically, the importance of this transmembrane protein in salt and water regulation is emphasized through loss-of-function (Pseudohypoaldosteronism type 1) and gain-of-function (Liddle's Syndrome) mutations of ENaC subunits found in Mendelian forms of hypo- and hypertension respectively (Rossier & Schild, 2008).

3.4.1 Liddle's Syndrome

Liddle syndrome (LS) is a disease described by a gain-of-function mutation in the ENaC. LS is an inherited illness characterized by uncontrollable hypertension due to

excessive sodium reabsorption (Knight et al., 2006; Rossier & Schild, 2008; Garrone et al., 2009; Bubien, 2010). The increase in salt absorption is attributed to the increased expression of ENaC at the cellular surface, creating an influx of sodium and water, resulting in an increase of blood volume. Over expression of ENaC in LS is due to mutation in the proline rich region of the C-terminal, intracellular PY motif (Knight et al., 2006; Garrone et al., 2009). The mutation results in the deletion of the PY motif that prevents binding to the regulatory *neural precursor cell expressed developmentally downregulated gene 4-like* (NEDD4L) protein (Figure 3). LS mutations are usually found in the β ENaC or γ ENaC units, where NEDD4L is unable to ubiquitinate these channel components, the indicator that signals them for degradation, or cellular recycling. This inability to down regulate ENaC subunits results in the accumulation of ENaC in the cell membrane and thus an increase in cross-membrane sodium transport and water reabsorption (Knight et al., 2006; Rossier & Schild, 2008; Garrone et al., 2009; Bubien, 2010).

3.4.2 Pseudohypoaldosteronism

Pseudohypoaldosteronism type 1 (PA1) is a rare form of hypotension, low BP, which mimics the effects of an aldosterone deficiency. PA1 is characterized by severe renal salt wasting, dehydration and hyponatremia (Kostakis, Cholidou & Perrea, 2012; Schild, 2004). This rare illness is the result of a mutation in at least one of ENaC's three subunits causing a truncated, or an abnormally structured protein, that impairs the proper function of the sodium channel. The non-functional ENaC are unable to transport sodium across ASDN and there is a loss of vital salts and water necessary in maintaining blood volume. Ultimately, both the gain- and loss-of-

function mutations illustrate the importance of ENaC regulation and its impact on BP.

3.5 Neural precursor cell expressed developmentally downregulated gene 4-like (NEDD4L)

NEDD4L is one of the nine-member family of Nedd4-like human ubiquitin ligases, a set of proteins that regulates biological signalling through ubiquitination. NEDD4L is classified as an E3 enzyme, meaning it catalyzes the final step of the transfer of ubiquitin (Ub) to the targeted substrate. NEDD4L is comprised of three main segments that facilitate this Ub transfer process, which identifies substrates for endocytosis and molecular recycling or degradation (Yang & Kumar, 2010). The homologous to E6-AP C-Terminus (HECT) domain is the active, or enzymatic ligase region of NEDD4L. The HECT domain is the portion that catalyzes the final transfer of Ub to the targeted substrate. Interaction of NEDD4L with the target substrate is facilitated with the four WW domains, which interacts with the PPxY(PY) motif of its target substrate (Figure 3). Additionally, NEDD4L has a calcium-sensitive (C2) domain that is responsive to intracellular calcium stimuli. The presence of calcium stimulus causes the NEDD4L regulatory proteins relocate to the cellular membrane facilitating ENaC binding and interaction. (Garrone et al., 2009).

The Nedd4 family is responsible in controlling a multitude of biological targets, though, ENaC is considered it's most famous with implications in Liddle's Syndrome. Further, the NEDD4L orthologue is regarded as the most potent ENaC regulator from the Nedd4 family (Dunn et al., 2002). The importance of ENaC in the regulation of BP has been well documented in the kidneys. However, NEDD4L and

other regulatory components are also found in tissues that ENaC has shown to be biologically functional. As previously discussed, recent evidence has shown the potential of a cerebral component that could be responsible for the SS phenotype (Huang, Veerasingham, & Leenen, 1998). This was explored further by Van Huysse, Amin, Yang, & Leenen (2012) in research using NEDD4L knockdown (-/-) mice. The NEDD4L (-/-) mice displayed a significant three-fold larger increase in BP compared to wild-type mice when intracerebroventricularly infused with a sodium-rich artificial CSF. This observation was then extinguished when the mice were infused with an ENaC blocker. Additionally, the NEDD4L (-/-) mice fed a high salt diet exhibited a significant increase in HR, BP, and a CSF with a high sodium concentration. This suggests that sodium concentrations coupled with the ENaC-NEDD4L relationship in brain regions can have a profound influence on BP.

The significance of NEDD4L on the regulation of ENaC and the potential of their combined importance in the SS phenotype has been demonstrated. However, due to the complex nature of NEDD4L the understanding of the ENaC-NEDD4L relationship continues to be problematic. Further investigations into the implications of NEDD4L in SS HTN are warranted.

3.5.1 The *NEDD4L* single nucleotide polymorphism rs4149601

The *NEDD4L* variation rs4149601, found located at the end of exon 1 is a guanine (G) to adenine (A) substitution reportedly found in 35.4% to 36.7% of Northern European descendants (dbSNPa, n.d.). The G-variant (the major allele) is considered a 'leaky splice' and can result in three protein products, two that include the C2 calcium dependent domain – Isoforms I and II – and a third (III) isoform that lacks

this C2 domain. However, the A-variant (the minor allele) can only splice isoform III, which is lacking the C2 domain, because it codes “AUA” instead of an “AUG” start codon necessary to transcribe the *NEDD4L* exons 2 through 7 that code the C2 domain. (Dunn et al. 2002; Garrone, et al., 2009). It has been proposed that this alternative spliced NEDD4L protein, lacking the C2 region (C(-)), could make it less susceptible to pressures from intracellular calcium concentrations, while the product with the C2 region (C(+)) is restricted by intracellular calcium levels, unlike the less regulated C(-) version, which is able to tag newly synthesized ENAC subunits and mobilized them for degradation (Itani et al., 2005; Garrone et al., 2009). A population-based study done by Fava, et al. (2006) on 4001 Swedish individuals demonstrated that the ‘GG’-genotype resulted in significantly (P=0.02) higher DBP and DBP progression (P=0.002), though, no significance was found with SBP. Their research shows that individuals with the G-variant present with higher BP, while the A-variant has some protective effect, a possible alleviation of the regulating function of NEDD4L on ENaC.

An observed increase of BP in relation with the G-variant of the *NEDD4L* rs4149601 SNP described in studies on Caucasian populations (Fava et al, 2006; Dahlberg, Nilsson, von Wowern, & Melander, 2007) has not been consistent across differing ethnic populations. For example, a ‘flip-flop’ effect has been observed in Chinese populations, where, contrary to the previous studies, the A-variant is described to be significantly associated with rising BP (Luo, et al., 2009; Zhao, et al., supplementary section 11.4). The potential implications of rs4149601 in SS HTN

and the inconsistent population-based findings makes this *NEDD4L* an attractive target for further research involving sodium and BP.

3.5.2 The *NEDD4L* single nucleotide polymorphism rs2288774

A second *NEDD4L* variation, rs2288774, is located on intron 6 and is a thymine (T) to cytosine (C) substitution that is found at frequencies from 43.8% to 51.8% in Europeans (dbSNPb, n.d.). Although, unlike rs4149601, no potential functional mechanism of the rs2288774 SNP has been identified it has been a study target for association studies involving BP. This rs2288774 SNP also often fails to produce a significant impact on BP when analyzed individually – A crude analysis by Liang et al., (2014) does describe a borderline significant ($P=0.044$) dominant model (TT vs CT+CC) without correction – though, significance is often found when analyzed in combination with another candidate region (Fava et al., 2006; Dahlberg, Nilsson, von Wowern & Melander, 2007; Wang et al., 2014). More specifically, the same above population study, by Fava, et al., (2006), indicated that ‘CC’ or ‘CT’ carriers had significantly higher ($P<0.04$) systolic and diastolic BP compared to ‘TT’ carriers when combined with the *NEDD4L* rs4149601 SNP. Additionally, while investigating salt-sensitivity (SS) in 39 subjects, Dahlberg, Nilsson, von Wowern and Melander (2007) found that there was a significant enhancement of systolic SS – higher BP in response to dietary salt – in subjects carrying both the GG-rs4149601 together with the CC-rs2288774 genotypes. Individually, it seems that the rs2288774 may not significantly impart effects on BP, however, with its possible synergistic effects with rs4149601 on BP and in augmenting SS, it emerges as a viable target for further investigation.

3.5.3 The *NEDD4L* single nucleotide polymorphism rs576416

Currently there is also no known functionality of the rs576416 SNP in the literature.

This third *NEDD4L* variation, rs576416, is a guanine (G) to adenine (A) substitution found at a frequency of 39.7% to 42.9% in European descendants (dbSNPc, n.d.).

The rs576416 SNP is from an Axiom Array Plate (see section 6.3) from an unpublished genome wide association study (GWAS) that is currently ongoing at the University of Ottawa Heart Institute in Ottawa, ON. Through the haploid analysis (Figure 4) of two Caucasian populations, the rs576416 was discovered to be in linkage disequilibrium (LD) with rs4149601. LD has been defined as “correlations among neighbouring alleles, reflecting 'haplotypes' descended from single, ancestral chromosomes” (Reich et al. 2001); that the two gene regions are dependent, or they get inherited/genetically transferred together to the offspring. It has been suggested that mapping out complex diseases, such as heart disease, hypertension, and diabetes, through LD projects can be beneficial and are crucial breakthroughs to reshape our thinking (Lander & Kruglyak, 1995). The rs576416 is an opportune target to further explore potential regions implicated in hypertension.

3.5.4 Study Purpose

The purpose of this study is to evaluate the impact of genetic variations of the *NEDD4L* gene – rs4149601, rs2288774, and rs576416 – on hypertension in Caucasian individuals. This is the first study aimed at elucidating the effect of three *NEDD4L* SNPs with age and sodium on hypertension in three Caucasian populations.

4.0 HYPOTHESES

- 1) The *NEDD4L* SNPs (rs4149601, rs2288774 and rs576416) genotypes will be associated with a rise in BP in response to increasing age and urine sodium levels.
- 2) The combination of the high risk genotypes from the *NEDD4L* SNPs will have an effect on BP according to increasing age and urine sodium levels.

5.0 OBJECTIVES

- 1) To investigate the influence of each *NEDD4L* rs4149601 and rs576416 SNP on BP response with increasing age and urine sodium levels in three hypertensive Caucasian populations from Canada, in Belgium, and Poland.
- 2) Investigate the influence of the *NEDD4L* rs2288774 on BP response with increasing age and urine sodium levels in the Canadian and Polish hypertensive populations.
- 3) To assess the relationship between the interaction of the *NEDD4L* rs4149601 and rs576416 genotypes and their effects on BP response with increasing age and urine sodium levels in the three hypertensive populations.
- 4) To assess the relationship between the interaction of the *NEDD4L* rs4149601/rs576416 and rs2288774 genotypes and their effects on BP response with increasing age and urine sodium levels in the Canadian and Polish hypertensive populations.

6.0 METHODS

6.1 Study Participants

Hypertensive subjects were obtained from the Flamengho study in Leuven, Belgium; and hypertensive patients were recruited in Ottawa, Canada, through the University of Ottawa Heart Institute by clinicians from the hypertension unit, and from the Institute of Cardiology in Warsaw, Poland.

For this project, only Caucasian participants were eligible to avoid the complication of ethnic influences on SS. SS is observed at a higher rate in black hypertensives (73%) than when compared with a white hypertensive group (56%)(Weinberger, 1996). The inclusion criteria for the hypertension group consist of: a family history (≥ 1 family member) of early onset hypertension (< 60 years old); a consistent resting BP of at least 130/85 mmHg; and a BP $< 160/100$ mmHg after stopping antihypertensive drugs for two weeks. 24hr ambulatory BP monitoring (ABPM) is used to confirm the subjects' BP status and to avoid white coat hypertension (Niiranen et al., 2014). Exclusion criteria includes: the presence of cardiovascular disease; diabetes; chronic kidney disease; alcohol overuse; regular NSAID use; and severe hypertension (BP >160 mmHg).

Research approval was achieved from the Ottawa Hospital Research Ethics Board. Subjects' informed consent was obtained prior to participation in the study. Blood samples were collected (see section 7.2) from the Canadian subjects, and the Polish team performed the DNA extraction of their samples in their laboratory facilities and then the DNA samples were shipped to Ottawa, Canada for genotyping and statistical analyses. The Belgium DNA samples were processed and genotyped

at Campus Sint Rafaël in Belgium, and their data was graciously made available for analyses. The subjects' complete volume of urine was collected for a 24-hour period for the urinary sodium, potassium and creatinine measures along with their height (cm) and weight (kg). Clinicians obtained serum sodium and potassium concentrations (mmol/L) from the subjects collected blood samples.

6.2 Sample Collection and DNA Extraction

Within the Canadian population, four EDTA blood tubes were collected, from each subject, by the clinical staff at the Ottawa Heart Institute. Three of the four blood tubes were then aliquoted into Ultident ProGene 2 mL screw cap tubes for DNA (buffy coat) extraction and enzyme assay (plasma, serum) studies. The fourth whole blood tube was reserved and stored along with the aliquots in -80°C freezer at the Ottawa Heart Institute. All of the patient samples were coded and tracked using the Specimen Management and Tracking System (SMTS). The research coordinator managed the corresponding demographic and clinical information.

DNA was extracted from the aliquoted buffy coat using the Qiagen Flexigene DNA extraction kit. The protocol used was for the isolation of DNA from 100-500 µL of buffy coat. The following modifications to the protocol were made: 500 µL of FG1 lysis buffer was used instead of 750 µL; 500 µL of FG2 denaturation buffer/Qiagen protease buffer mix was used instead of 300 µL; 500 µL of 100% isopropanol was used instead of 300 µL; 500 µL of 70% ethanol was used instead of 300 µL; 100 - 200 µL of the FG3 hydration buffer was added. The Nanodrop 2000 Spectrophotometer (Thermo Scientific) was used to check the DNA concentration and its purity. The DNA concentration was calculated using the following equation:

$$[\text{DNA}] = \text{OD}_{260} \times \text{Dilution factor} \times 50 \text{ ng}/\mu\text{L}$$

where [DNA] is in ng/ μL and 50 ng DNA/ μL = 1 OD₂₆₀ unit.

The DNA purity was determined using the ratio of absorbance at 260 nm and 280 nm. A sample is considered to be pure and free of proteins and RNA when the A_{260nm}/A_{280nm} is around 1.8. Blood collections for the Belgian and Polish populations were done at their respective institutions following similar protocols.

6.3 *NEDD4L* genotyping

DNA samples from the Canadian (202 hypertensives) and Polish (103 hypertensives) populations were processed on the GeneTitan at the Ottawa Heart Institute. The GeneTitan uses the automatic Affymetrix Axiom platform (Axiom® Genome-Wide CEU 1 Array Plate) to genotype 526,549 Caucasian-specific SNPs per DNA sample. This particular Array plate included 104 different *NEDD4L* SNPs – excluding rs4149601 and rs2288774 that were genotyped via TaqMan MGB probe-based RT-PCR methods (see section 6.3.1). SNPs and individual samples are excluded from GWAS analysis if they deviate from the Hardy Weinberg equilibrium, the allele frequency naturally expected in a population, in the samples at $p < 0.0001$, or yield an individual SNP rate $< 95\%$, to ensure genotyping accuracy. The Belgium hypertensives were individually genotyped for 13,659 SNPs, on a separate technological platform at the Campus Sint Rafaël in Belgium. Six hundred and thirty four ($n=662$) hypertensive individuals – 190 from Canada, 95 from Poland, and 377 from Belgium – passed the quality control portion of the GWAS analyses.

6.3.1 TaqMan MGB probe-based RT-PCR

The TaqMan MGB probe-based RT-PCR genotyping technique was used because the *NEDD4L* rs4149601 and rs2288774 SNPs of interest were not available on the GWAS array used at the University of Ottawa Heart Institute. It allows for easy and accurate genotyping using fluorescent readings (Leutenegger, 2001). Unique SNP primers are labeled separately with FAM and VIC fluorescent tags along with fluorescent quenchers (MGB) – the fluorescent inhibitor (Applied Biosystems). SNP Primers and TaqMan MGB probes (assay #s C_1424558_10 for rs4149601 and C_15879708_20 for rs2288774) synthesized by Applied Biosystems are utilized for amplification following the standard recommendations from Applied Biosystems. During the process of DNA replication, the fluorescently tagged primers are implemented into the sequence being amplified and the fluorescent quencher region of the primer is cleaved, allowing for the newly formed DNA to fluoresce the wavelength associated with the SNP, which is then read by the Bio-Rad CFX96 system (Figure 5). Allelic discrimination and analysis of data from end point fluorescence measurements are used to convert allelic calls to genotypes using CFX96 real-time system and the Bio-Rad CFX Manager software 3.1.

6.3.2 Polymerase Chain Reaction (PCR), Sequencing PCR and DNA Sequencing

If genotyping with the TaqMan MGB probe is unsuccessful then sequencing is performed to determine the genotype. The following describes the gene sequencing for the *NEDD4L* SNP regions:

Primers for the PCR reaction for both the exon SNP rs4149601 and intron SNP rs2288774 were selected from the literature (Fava et al., 2006). The forward primer sequence for rs4149601 is TGCAGCCACGACTTCCGCAT and the reverse primer

sequence is ATCAGGCCTGGTTAGACTTGTTCCCT. As for rs2288774, the forward primer sequence is ACAGTCTCATGTTTGATGCTTCGT and the reverse primer sequence is ACGTGCTTCATTTTCAGCCTTCA. The Promega PCR protocol is followed using the specified melting temperature (T_M) of the primers - 55.9 °C, 57.7 °C, 54.0 °C, and 53.0 °C respectively – for the rs4149601 (exon) or rs2288774 (intron) SNPs.

PCR (Eppendorf Mastercycler Gradient PCR machine) is used to amplify the region of interest before running the sequencing PCR. 1% agarose gel (Invitrogen) with 4 μ L of ethidium bromide (Invitrogen; [10 mg/mL]) is run on the Thermo Scientific Owl EasyCast™ B2 for 40 minutes at 100 V to confirm the amplification of the target region. The gel is visualized using the Alpha Innotech Alpha Imager Mini (Fisher Scientific) and O'Gene DNA Ruler (Thermo Scientific) 50bp and 100 bp ladders are run along with the PCR samples for reference.

After running the normal PCR and confirming that the target region is amplified, sequencing PCR is performed (Eppendorf Mastercycler Gradient PCR machine) using ddNTPs associated with particular fluorescent dyes (França, Carrilho & Kist, 2002). The mastermix includes BigDye® Ready Reaction premix [1X], the BigDye® Sequencing buffer [1X], forward or reverse primer (3.2 pmol), water, and genomic DNA (5-10 ng) for a total volume of 20 μ L.

The BigDye® Terminator Cycle Sequencing Kit protocol by Applied Biosystems is followed.

After the sequencing PCR, the DyeEx 2.0 Protocol for Dye-Terminator Removal (Qiagen) is used to prepare the samples for the sequencing machine. Samples will be subjected to forces at 800x g to elute the DNA out. DNA is then dried

at 95°C for 15-20 min using a heat block. 20 µL of formamide, a thermodenaturing agent, is then added to the dried DNA and it is incubated for an additional five minutes on the heat block at 95°C. The samples are transferred to sequencing tubes within the sequencing machine (Perkin Elmer ABI Prism 310 Genetic Analyzer) for analysis. The DNA – containing the fluorescent ddNTPs – migrates through a capillary, due to an applied electrical potential, towards the positive electrode. The migration speed of the ddNTPs is affected by size; the smaller ddNTPs migrate much more quickly than the larger ddNTPs. A laser is used to stimulate the fluorescent ddNTPs that is captured by a fluorescence detector, which produces a graphical read out of the DNA sequence.

6.4 Statistical Analyses

Differences in demographic characteristics between the three hypertensive populations was tested using a one-way analysis of variance (ANOVA) for continuous variables and a chi squared (χ^2) test of homogeneity (Hogg, Tanis & Zimmerman, 2015) for categorical variables. The ANOVA and χ^2 population comparison and Hardy-Weinberg equilibrium analyses were performed using SPSS software where any P-value below 0.05 was considered significant. Simple and multiple linear regression analyses measuring the effect of variables on blood pressure, SBP and DBP respectively, were analyzed using the Statistical Analysis System (SAS Institute, Cary, North Carolina, USA) 9.0.3 software, specifically the 'PROC REG' procedural code (see section 11.3). Importantly, the "VIF" and "COLLIN" model statements were used to assess collinearity of clinical and demographic variables, and the "ACOV" model statement to adjust statistical models for

heteroscedastic population distributions, since the variation demonstrated in BP increases with age (see Figure 6). A P-value below 0.05 was considered to statistically reject the null hypotheses: that there is no relationship between BP and the genotypes of the three *NEDD4L* SNPs with increasing age and urine sodium.

6.5 Abbreviated methods for the rs4149601 meta-analysis article (see 11.3.3)

A systematic literature search for *hypertension* and (*NEDD4L* or *NEDD4-2* or *NEDD4.2* or *RSP5* or *KIAA0439* or *rs4149601*) and (*polymorphism* or *SNP* or *variation* or *mutation*) was done in the PubMed (MEDLINE), EMBASE, and the China National Knowledge Infrastructure (CNKI) databases. Studies were considered eligible if the included the following: 1) Case control, cohort, or cross sectional study; 2) a clinical diagnosis of essential hypertension; 3) the study reported specifically on the association of rs4149601 on essential hypertension; 4) was not a molecular or animal study; and, 5) was not a review, commentary/editorial, or conference abstract. A meta-analysis was conducted using the Review Manager 5 (version 5.3.5) software. No *a priori* genetic model was assumed and comparisons of odds ratios and 95% confidence intervals (CIs) were used. The complete methodology can be found in section 11.3.3. My contributions to the final format of this article included: updating the most recent literature search of the three databases; completing the Prisma Checklist required for meta-analyses; and, conducting a critical review of the article prior to submission.

7.0 RESULTS

7.1 Demographic/Clinical Data

A total of 667 hypertensive individuals of Caucasian ancestry were successfully genotyped from Ottawa, Canada (n=190), Leuven, Belgium (n=380), and Warsaw, Poland (n=97). Five samples were removed from the analysis by clinicians at the Ottawa Heart Institute – three for having extreme BMI measures and two for abnormally high DBP (above 100 mmHg). The clinical characteristics of the remaining 662 subjects included for analysis, and their stratification by location and sex, are summarized in Table 1.

The variables shown in Table 1 were selected, as previously mentioned (section 4.2), because of their implications on BP. The population, when proportioned by location, was significantly different (ANOVA $P < 0.05$) across all the clinical variables. More specifically, the Belgium and Polish hypertensives have a higher proportion of males (72.4% and 75.8%) when compared to those from Canada (63.2%). The divergent distribution of males and females led to further stratification of the hypertensive population by sex. Though, even with the separation by sex, the inconsistency of the clinical characteristics across the three populations persisted (ANOVA $P < 0.05$), with the exception of SBP ($P = 0.079$) and the urine ratio for sodium and potassium ($P = 0.159$) within the female subset, which led to the male and female hypertensives being analyzed separately. The males and females from Canada were substantially older (M: 47 ± 9 yrs; F: 51 ± 8 yrs) and had on average a greater BMI (M: 29.4 kg/m^2 ; F: 28.2 kg/m^2) than those hypertensives from the Belgium (M: 39 ± 11 yrs, $26.5 \pm 3.8 \text{ kg/m}^2$; F: 43 ± 11 yrs, $25.8 \pm 4.3 \text{ kg/m}^2$) and

Polish populations (M: 39±11 yrs, 27.2±3.5 kg/m²; F: 41±9 yrs, 24.0±4.7 kg/m²). Both the males and female hypertensives from Canada also displayed the highest SBP (M: 140±9 mmHg; F: 139±10 mmHg), when the Belgium sexes exhibited the lowest DBP (M: 85±8 mmHg; F: 86±6 mmHg). These Belgian groups also had the greater levels of blood sodium (M: 141.9±2.8 mmol/L; F: 141.1±4.4 mmol/L) and urine sodium (M: 142±47 mmol/day; F: 150±54 mmol/day). The Polish hypertensives had the most elevated blood potassium levels (M: 4.4±0.5 mmol/L; F: 4.2±0.3 mmol/L) against the Canadians (M: 4.0±0.3 mmol/L; F: 4.0±0.3 mmol/L) and Belgians (M: 4.1±0.3; F: 4.0±0.3), though their urine levels of potassium were lesser (M: 43.7±18.9 mmol/L; F: 31.6±9.9 mmol/L). There is a considerable amount of heterogeneity between the clinical characteristics across the populations by both location and sex. The populations are not homogenous and thus will be stratified by location and sex for the analyses.

7.2 Abbreviated results from the rs4149601 meta-analysis article (see section 11.3.3)

The systematic review identified 71 studies that fit the eligibility criteria. From these, 5 studies of 4531 East Asian individuals (n=2093 hypertensives and n=2438 normotensive controls) were selected for analysis. A significant recessive model was identified where AA-rs4149601 individuals were 63% more likely to present with essential hypertension than GA- and GG-ra4149601 subjects (OR=1.63 95% CIs: 1.2019-2.21. P<0.01). The complete results can be found in section 11.3.3.

7.3 Blood pressure progression with age

A well reported general trend in the population is the progression of BP with age (Vasan et al., 2002; Leenen, et al., 2008; Stats Canada, 2014). The results of the

simple linear regressions of age versus SBP and DBP in the stratified populations are available in Table 2. The Canadian males ($\beta=0.244$ mmHg/year, $P=0.002$) females ($\beta=0.356$ mmHg/year, $P=0.0118$) showed significant increases in SBP with regards to age (Figure 6), similarly in the Belgian males ($\beta=0.090$ mmHg/year, $P=0.0191$). However, the Belgian females, and both the Polish males and females did not exhibit a significant age increase in SBP ($P>0.05$). The males from Canada ($\beta=0.162$ mmHg/year, $P=0.0122$) and Belgium ($\beta=0.310$ mmHg/year, $P<0.001$) were the only groups that reflected the general trend of a BP increase with age for DBP. The Polish males and females from each location did not show a significant relationship between age and SBP ($P>0.05$). The Canadian and Belgium males were the only two populations to match the assumption that age is associated with an increase in SBP and DBP, as mentioned, is seen in the general population. The Polish population and the female hypertensives from Canada and Belgium did not present with an age related effect on BP. These groups are subsequently removed from the analysis, as they do not appropriately reflect the general population since they do not display the well-described age associated increase in BP seen in the literature. The multivariate regression analyses will focus on the Canadian male and Belgium male hypertensive populations.

7.4 Multivariate linear regression analyses

The genotype frequencies for the three SNP's (rs4149601, rs2288774, and rs576416) in the Canadian male and Belgium male groups do not deviate from the Hardy-Weinberg equilibrium ($P>0.05$). The *NEDD4L* MAF are also similar to frequencies reported in the 1000 Genome project for European descendants

(n>100) – ‘A’ rs4149601 MAF was 33.8% for the Canadian, and 35.2% for the Belgian hypertensives versus 35.4%-36.7% (dbSNPa, n.d.); a MAF for ‘A’ rs576416 of 42.4% in Canada and 36.8% in Belgium matched to 39.7%-42.9% (dbSNPb, n.d.); and for ‘C’ rs2288774 a MAF of 46.3% in the Canadian hypertensives compared to 43.8%-51.7% (dbSNPc, n.d.). The two populations, the Canadian and Belgian males, have met the assumptions of increasing BP with age, no deviation from the HWE, and similar MAF’s to other European descendants. These hypertensive subsets will now be analyzed, using a multivariate linear regression modeling, to examine the influence of age, BMI, blood sodium, blood potassium, the levels of urine sodium and potassium corrected for creatinine, the urine ratio of sodium and potassium, and the three NEDD4L genotypes on both SBP and DBP.

7.4.1 Multivariate regression analyses of clinical variables on blood pressure

The results of the multiple linear regression analyses, using the clinical characteristics, in the two male hypertensive populations from Canada and Belgium are presented in Table 3. The overall regression model in the Canadian males fails to detect a significant effect of the factors on the variations found in SBP ($P>0.05$). The male regression model for DBP in the Canadian males significantly explained a portion of the variance ($R^2=0.1409$) in DBP, a significant increase associated with age ($\beta=0.178$ mmHg/year, $P=0.0103$) and drop with blood potassium levels ($\beta=-3.78$ mmHg/mmol/L, $P=0.039$). The remaining variables were not found to individually impact the variability of DBP ($P>0.05$). The full regression models for the Belgian males were significantly attributable to the changes found in SBP ($P=0.0176$) and DBP ($P<0.0001$). Though, the significant effect from age was no

conserved in the SBP model, where only BMI had individual influence ($\beta=0.375$ mmHg/kg/m², $P=0.0045$). Age was the only component to show significance in the Belgian DBP regression model ($\beta=0.291$ mmHg/year, $P<0.0001$). The loss of significance of the overall Canadian model, and the same for the age variable in the Belgian SBP model, paired with the models relatively low ability to account for variance ($R^2=0.1087$ and $R^2=0.0624$ respectively), created an issue for future analyses using the *NEDD4L* SNPs. A decision was made to simplify our models, and to identify the more significant, influential variables through backwards regression modeling.

7.4.2 Backwards regression analyses of clinical variables on blood pressure

A step-wise backwards regression model will take the full model, which includes all the variables, and reduce it into a simpler statistical model that is more stringent. Computationally, in a step-by-step process, the least significant variable from the full model will be removed and the model is refit such that it is looking for the reduced model that best represents (lower P-value and higher R^2) the variations found in the populations BP. The outcomes of the backwards regression model are represented in Table 4. The simplified model for SBP in the Canadian hypertensive males includes age (years), blood potassium concentration (mmol/L), and the amount of sodium excreted in the urine, corrected for creatinine levels (mmol/day). These same variables were included in Canada's DBP model with the addition of BMI (kg/m²). Age, BMI, and corrected urine sodium were the three elements included in the simplified model for the Belgian males for both SBP and DBP. These simplified models are used in the genotype analyses.

7.4.3 The *NEDD4L* rs4149601, rs576416, and rs2288774 multivariate regression analyses on blood pressure

The three *NEDD4L* SNP's, rs4149601, rs228774, and rs576416, are included into the simplified regression models identified in section 7.3.2 for the Canadian male hypertensives. The Belgian males only have genotype data for the rs4149601 and rs576416 SNPs, as the DNA was unavailable for rs2288774 genotyping. The genotype effect of the individual SNP's, as well as their coupled interaction, in combination with age and corrected urine sodium are presented in the following analyses.

7.4.3.1 The multivariate regression analyses on the effect of the interaction of age and urine sodium, with the individual *NEDD4L* rs4149601 and rs576416 SNPs, on SBP and DBP

Analyzing the effect of the individual *NEDD4L* genotypes – rs4149601 and rs576416 – and the interaction with both age, urine sodium on BP in the Canadian and Belgium male hypertensives are summarized in Tables 5 through 10. Table 5 contains the multiple regression modeling on the Canadian male hypertensive population where the rs4149601 SNP was not found to significantly influence SBP (model $P=0.1342$). The significant model on DBP ($P=0.0200$) describes a negative relationship of blood potassium ($\beta=-4.78$, $P=0.0063$) on DBP, however, none of the other variables significantly influence DBP ($P>0.05$). The summary of the modeling of the rs4149601 SNP with age and urine sodium on males from Belgium is available in Table 6. For SBP, all of the variables positively influence BP – Age ($\beta=0.671\text{mmHg/year}$, $P=0.0004$); BMI ($\beta=0.332\text{mmHg/kg/m}^2$, $P=0.0065$); urine sodium ($\beta=0.199\text{mmHg/mmol/day}$, <0.0001); GA-rs4149601 interacting with age

and urine sodium ($\beta=0.00369$ mmHg/[year*mmol/day], $P=0.0129$); and GG-rs4149601 interacting with age and urine sodium ($\beta=0.00463$ mmHg/[year*mmol/day], $P=0.0082$). The regression for DBP in the Belgian males only displays a significant age effect on BP ($\beta=0.818$ mmHg/year, $P=0.0073$); there is no interaction effect from the rs4149601 genotype, with age and urine sodium.

An analysis of the rs576416 *NEDD4L* SNP, identified to be in LD with rs4149601 from the unpublished GWAS, with BP is summarized in Table 7 and 8. The model reflecting the variation displayed by SBP in the Canadian males (Table 7) failed to significantly explain the variations observed in their SBP, while the model representing the changes in DBP ($R^2=0.1904$) was only significantly explained by blood potassium levels ($\beta=-4.15$ mmHg/mmol/L). The variation ($R^2=0.1082$) of SBP model in the Belgium males (Table 8) is significantly accounted for by each variable – Age ($\beta=0.504$ mmHg/year, $P=0.003$); BMI ($\beta=0.316$ mmHg/kg/m², $P=0.0127$); corr.uNa⁺ ($\beta=0.110$ mmHg/mmol/day, $P=0.0055$); and GA-rs576416 interacting with age and urine sodium ($\beta=0.00334$ mmHg/[year*mmol/day], $P=0.0131$) – with the exception of the interaction of the GG-rs576416 genotype with age and urine sodium ($P>0.05$). In similar fashion to rs4149601 model, age is the only significant contributor ($\beta=0.646$ mmHg/year, $P<0.0001$) to DBP variation ($R^2=0.2440$) when analyzed with the rs576416 genotypes.

7.4.3.2 Age and corrected urine sodium interaction with the high risk *NEDD4L* rs4149601 and rs576416 genotypes on SBP and DBP

The GG-rs4149601 was found to significantly influence SBP in Belgian males (section 7.3.3.1; Tables 5 & 6), and previous research, in Caucasian population

samples (Fava et al, 2006; Dahlberg et al. 2007), also identified the GG-rs4149601 genotype with higher DBP as well as resulting in BP more susceptible to SS. The GG-group will be regarded as the high risk genotype for rs4149601.

The case with selecting the high risk genotype for rs576416 is less obvious, as there is no literature on its influence on BP, and our modeling (section 7.3.3.1; Tables 7 & 8) did not provide any conclusive outcomes. The 'A' allele, or AA-genotype will be selected as the "high risk" genotype for our analyses based on the following interpretation. The Belgium SBP model shows a significant difference between the GA-rs576416 from the intercept, that includes the GG-rs576416 ($P=0.0130$). This suggest the potential influence of the A allele on SBP. The AA-rs576416 was not significantly different from the intercept ($P=0.5678$) but it had a similar magnitude of effect on BP as GA-rs576416 ($\beta= 0.00241$ mmHg/[year*mmol/day] vs $\beta=0.00334$ mmHg/[year*mmol/day] respectively) (Table 8). A similar model in the Belgium males (Supplemental Table 1; section 11.4.1), using the AA-rs576416 as the intercept, shows no significant differences of either the GG- or GA-rs576416 from the intercept but had directional and magnitude differences ($\beta= -0.00241$ mmHg/[year*mmol/day] vs $\beta=0.000931$ mmHg/[year*mmol/day] respectively) (Supplemental Table 1, section 11.4.1). This could suggest that the AA- and GA-rs576416 genotypes have a more similar influence on SBP than the GG_rs576416. The AA-rs576416 will be used as the high risk genotype for further analyses.

A combination of the *NEDD4L* GG-rs4149601 and AA-rs576416 genotypes with age and corrected urine sodium against BP is described in Tables 9 and 10. These complex models failed to illicit any interaction effects on DBP progression, though blood potassium significantly explains some variation in DBP ($\beta=-3.86\text{mmHg}/\text{mmol}/\text{L}$, $P=0.0229$). The model for SBP in the Canadian males (Table 9) is borderline significant ($P=0.0558$). Within the model there is a significant effect, once again, from blood potassium ($\beta=-5.28\text{mmHg}/\text{mmol}/\text{L}$, $P=0.0466$) as well as a high order interaction from age, corrected urine sodium and the two high risk genotypes – GG-rs4149601 and AA-rs576416 – on SBP ($\beta=0.0210\text{mmHg}/[\text{year}*\text{corr.uNa}+*\text{rs4GG}*\text{rs5AA}]$, $P=0.0366$). The SBP and DBP regression models in the Belgium males both fail to find a significant high order interaction effect of the GGrs4149601 and AA-rs576416 genotypes ($P>0.05$) with age and corrected urine sodium. The models also show a significant effect from BMI ($\beta=0.328$, $P=0.0114$) and borderline line significant effect from age ($\beta=0.195$, $P=0.0737$) on SBP; and, a significant effect from age ($\beta=0.391$, $P=0.0043$) and borderline line significant effect from BMI ($\beta=0.235$, $P=0.0670$) on DBP.

7.4.3.3 The effect from the interaction of age and urine sodium, with the individual NEDD4L rs2288774 on SBP and DBP in males from Canada

The genotyping of *NEDD4L* rs2288774 was only available in the Canadian male population. The analysis of rs2288774 and its effects on SBP and DBP are presented in Table 11. There was no statistical significance found from modeling the individual rs2288774 genotypes on SBP, though, the analysis on DBP was borderline ($P=0.0504$). BMI ($\beta=-3.08\text{mmHg}/\text{kg}/\text{m}^2$, $P=0.0374$) and blood potassium ($\beta=-4.48\text{mmHg}/\text{mmol}/\text{day}$, $P=0.0224$) were the factors within the model to have a

significant impact on DBP – Age, corr.uNa⁺, and the interaction of age, urine sodium with the rs2288774 genotype were non-significant (P>0.05).

7.4.3.4 Age and corrected urine sodium interaction with the high risk NEDD4L rs4149601 and rs2288774 genotypes on SBP and DBP in males from Canada

The effect of the NEDD4L GG-rs4149601 and CC-rs2288774 genotypes combination with age and corrected urine sodium on BP is available in Table 12. The model analyzing the effect of this complex interaction on DBP is significant (P=0.0355), though, the only significant variable within the model is blood potassium (β =-4.04mmHg/mmol/L, P=0.0246). The high order interaction of the two *NEDD4L* genotypes – GG-rs4149601 and CC-rs2288774 – with age and corrected urine sodium is significant in the SBP model (β =0.0201mmHg/[year*corr.uNa⁺*rs4GG*rs2CC], P=0.0414); however, the overall model is not significant at 5% (P=0.0939). No other variables are significant in this model with SBP.

7.4.3.5 Age and corrected urine sodium interaction with the high risk NEDD4L rs2288774 and rs576416 genotypes on SBP and DBP in males from Canada

The evaluation of age and urine sodium corrected for creatinine combined with the *NEDD4L* CC-rs2288774 and AA-rs576416 genotypes are summarized in Table 13. The overall model accounting for variations in SBP fails to explain significantly changes attributed to SBP (P=0.1141), and no variables show any significant effects within the model (P>0.05). Blood potassium significantly accounts for some of the variations appreciated by DBP (β =-4.44mmHg/mmol/L, P=0.0077) in the complex model (P=0.0068) including the multiple SNP interaction – between CC-rs2288774 and AA-rs576416. Age, BMI, and corr.uNa⁺ were also non-significant factors on DBP.

8.0 DISCUSSION

Susceptibility to dietary sodium has long been postured as an accessory to increasing BP (Appel & Whelton, 2013). Reducing sodium consumption has proven to be effective at moderately reducing BP (He & MacGregor, 2002; Bray et al., 2004). However, salt-sensitivity (SS), a characteristic not uniformly observed across the population (Tesson & Leenen, 2007) has been suggested to not only exist at higher rates in the hypertensives, but could also be subject to age-related changes (Weinberger & Fineberg 1991). It has been proposed that the influence of dietary sodium on BP increases with age (Dahlberg et al., 2007). Previous works by our group (Zhao, et al., supplementary section 11.4) and others (Huang, Van Vliet, & Leenen, 2004; Huang, Amin, & Leenen, 2006) have demonstrated that *NEDD4L* could play a potential role in this process.

The robust analysis by Zhao et al. (supplementary section 11.4), evaluating the *NEDD4L* rs4149601 SNP on essential hypertension, found the *NEDD4L* rs4149601 SNP to be significantly associated with this phenotypic presentation. This meta-analysis on 2093 hypertensive East Asians and 2438 normotensive controls held the regressive model, the AA-rs4149601, to significantly increase the risk of HTN (summary OR=1.63, 95% CI: 1.20-2.21, $p < 0.01$). The included studies were identified by systematic review, and lent some limitations. The findings of this study only included East Asian subjects and cannot be extrapolated onto other ethnic groups, as the G-rs4149601 variant has been associated with increasing BP in Caucasian populations (Fava et al., 2006; Dahlberg et al., 2007). Furthermore, blood pressure measurements were not reported using 24 hour monitoring, leading to the

risk of misclassification of HTN. As well, sodium was not a focus in the studies identified by the review, though, previous research has found sodium (Dahlberg et al., 2007) and age-sodium related changes (Weinberger & Fineberg 1991) with regards to NEDD4L. However, the impact of all NEDD4L, age, and sodium has not been investigated at the population level.

This current study aimed to evaluate the influence of age and sodium paired with previously identified *NEDD4L* rs4149601 and rs2288774 SNPs (Fava, et al., 2006), and the novel loci rs576416 on BP at the population level in three hypertensive groups from Ottawa, Canada; Northern Belgium (the Flemish Study on Environment, Genes and Health Outcomes); and, Warsaw, Poland.

From this cross-section, observational study design, a total of 662 hypertensive Caucasian individuals were included from Canada (n=190), Belgium (n=377), and Poland (n=95). The three hypertensive populations had comprehensive differences across all clinical variables (ANOVA $P < 0.05$) preventing a pooled analysis. The dissimilarities between the populations could be partially due to disparities in sex proportions. The two European populations had recruited more males than the Canadian hypertensive group. General population trends (Stats Canada, 2014) have shown men to be more susceptible to hypertension at younger ages than pre-menopausal women (Yanes & Reckelhoff, 2011; Maranon & Reckelhoff, 2013) and could explain some of the differences found across the three populations. After stratifying the populations by sex, however, the significant differences across the majority of clinical variables in the three populations continued to persist – except for SBP and the ratio between urine sodium and

potassium concentrations in females (Table 1). Although, stratification by sex was unable to rectify any of the disproportions observed between the three hypertensive populations, it remained sufficiently justifiable to stratify by both location and sex for the regression analyses against BP.

The strict inclusion criteria (see section 7.1) implemented in order to protect from spurious genetic ethnic effects (Weinberger, 1996) and minimize the misclassification of the hypertensive phenotype with 24 hour ABPM (Khan et al., 2007; Pickering, Eguchi & Kario, 2007) was considered a strength of this study's methodology. However, this approach could have been susceptible to unforeseen cross-cultural, and recruitment challenges. Heterogeneity has previously been described as a consequence of multinational studies as recruitment rates can vary per geographic region, but also cultural differences can impact variables (Berthon-Jones et al., 2015; Grill et al, 2015). For example, European populations generally ingest on average more dietary sodium than North American's (Wolf-Maier et al., 2003; Powles, et al., 2013). It is foreseeable that other differing cultural practices, that have been associated with effecting BP, not measured in this study, such as physical activity (Cornelissen & Smart, 2013) and smoking status (Jatoi, Jerrard-Dunne, Feely, & Mahmud, 2007), could also be a source of inter-population variability. Additionally, difference in strategies for patient recruitment could have contributed to the variations observed between the three hypertensive populations. Particularly, in Ottawa, Canada an active recruitment campaign was used to inform the public of the study in the form of radio and public transportation advertisements. These approaches could also be susceptible to cultural differences,

and result in sampling variations between the three recruitment centers. Furthermore, the Canadian Caucasian hypertensives are likely a more heterogeneous population than the Belgian and Polish groups. Canada was founded, and continues to be settled by diverse populations, which leads to the increased possibility of mixed ethnicities that can compound the previously identified differences between our Caucasian hypertensive groups.

The progression of BP with age is an important assumption that is habitually described in the general population (Franklin et al., 1997; Liu et al., 2001; Leenen et al., 2008; Leenen, McInnis & Fodor, 2010; Stats Canada, 2014). Simple age regressions with BP were executed on each population subset, once stratified by location and sex. Only the Canadian and Belgium males found a significant ($P < 0.05$) association between age and both SBP and DBP. The Polish males displayed a positive BP trend versus age (Table 2) though they lacked significance (SBP: $P = 0.1333$; DBP: $P = 0.0874$), which is likely due to less statistical power ($n = 72$). The Belgian and Polish females both also fail to observe a relationship in a simple regression between age with either SBP or DBP. The absence of a trend with BP could be the result of the young average age of the females in the Belgian (Age = 43 ± 11 years) and Polish (Age = 41 ± 9 years) subsets from the population (Table 1). As already mentioned, pre-menopausal women are protected from developing the hypertensive phenotype, when compared to men (Yanes & Reckelhoff, 2011; Maranon & Reckelhoff, 2013). Gold et al. (2001) observed the average onset of menopause after the age of 51 years, which is followed by a striking

increase in the prevalence of hypertension in females (Yang & Reckelhoff, 2011). The younger females in the Belgian and Poland populations could remain below the threshold for displaying a significant increase in BP across their age range. In comparison, the female hypertensives from Canada show a significant association with SBP progression, but not with DBP. Thus, the Polish population, and the females from Canada and Belgium do not meet the assumption of increasing BP with age and are subsequently unsuitable for the multiple regression analyses with the *NEDD4L* genotypes and sodium.

Comparatively, the hypertensive males from the Canadian (n=120) and Belgium (n=273) subsets were found to demonstrate a positive age association with BP in the simple regression analyses. A preliminary full multiple regression model, with the complete set of variables was run within each population subset versus SBP and DBP. The inclusion of the complete set of clinical variables does not improve the model fit found in the simple regression with age. Significance is lost when modeling SBP with the Canadian males subset, and the significance of age is lost in the SBP model for the Belgium male subset. Additionally, the analyses of the full models do not seem to better represent the variations (R^2) observed from the simple regressions with age. The loss of significance paired with the methodology of loosening model fit, with regards to the R^2 – a measure of how close the linear model reflects the data (Quinn, G. & Keough, 2002; Motulsky, 2014) – suggests the full model is not an improvement. The additional variables in the full model do not seem to add any value in terms of describing the variance, nor improving the significance of the model observed with age in both SBP and DBP. Although, the simple age

regression appears stronger at describing BP than the full model, the full model still highlights several factors that approach, or show a significant influence on BP, and they should not be ignored.

A backwards-stepwise selection regression is a computational method that begins with the full model, including all of the independent variables, and it sequentially removes independent variables that least contribute to the model on BP (Quinn, G. & Keough, 2002; Motulsky, 2014). The backwards regression method was applied to both the Canadian and Belgium males, SBP, and DBP separately due to the population differences discussed previously. The reduced model is based off of the results from the backward regression modelling (Table 4). An obvious limitation of the stepwise regression is that the investigators do not have any control over what variables get eliminated (Quinn, G. & Keough, 2002; Motulsky, 2014). As seen with both male population subsets, the variables of interest to the hypothesis – Age and urine sodium – were added to the outcome of the backward regression (Table 4). The reduced models are applied to the hypertensive subsets with both corrected urine sodium and age included in order to evaluate their effects in combination with the *NEDD4L* SNPs on BP.

The modified models that evaluated the effects of the select variables from the backwards regressions – Age, BMI, blood potassium and corrected urine sodium concentrations – with the three *NEDD4L* SNPs, rs4149601, rs2288774 and rs576416, on BP were unable to provide any consistent findings. The individual evaluations of the loci only demonstrated a single, positive, significant association of

the GG- and GA-rs4149601 with SBP in the Belgium males. The individuals with the 'G' rs4149601 allele had a higher SBP measurement due to age and urine sodium when compared to the Belgium hypertensives with the 'AA' variant. This finding similarly reflects the genetic relationship of 'G' carriers with increased BP and SS described by Dahlberg et al. (2007) and Fava et al. (2006). However, though excited of this positive relationship on supporting previous work, our advancement must remain both critical and cautious. The lack of a relationship of rs4149601 in the Canadian heeds such a response, as other studies have also described issues with replication. Research by Lin et al. (2007) provided important insight into how genotypes can theoretically present as high risk in one population, yet be protective in another. Furthermore, studies on a both Chinese (Wang et al. 2014) and Caucasian sample (Russo et al. 2005) have described a genetic flip-flop effect within their respective populations. These flip-flop mechanisms are still yet to be understood, but it has been proposed that studies of multiple locations, multiple genetic loci that contribute to disease, and even LD variability could contribute to such a phenomenon (Wang et al., 2014).

The novel *NEDD4L* rs576416 SNP was identified as being in LD with rs4149601 from the unpublished GWAS data conducted at the University of Ottawa Heart Institute (Figure 4) on the Canadian and Polish populations. A positive influence was found for the GA-rs576416 on SBP in the Belgium hypertensive population subset. The 'A' variant was selected as the 'high-risk' rs576416 allele (section 7.3.3.2) for the purpose of evaluating the interaction of rs576416 with the GG-rs4149601 high risk genotype previously identified (Fava et al., 2006; Dahlberg

et al., 2007). The AA- and GA-rs576416 slopes against SBP did not differ significantly (Supplemental table 1), while the GA-rs576416 regression slope was significantly larger ($P=0.0131$) than the GG-rs576416 against SBP (Table 8). Even with the lack of significance for the rs576416 'AA' variant remains biologically interesting since the magnitude of the interaction is similar to that of the 'GA' genotype when compared to the 'GG' group.

A design aspect of our study was to evaluate in combination the three *NEDD4L* genotypes against BP. Analyzing multiple genetic loci, increasing the number of regions analyzed, would potentially help alleviate some of the suggested impact of a flip-flop phenomenon suggested by Lin et al., (2007). None of the *NEDD4L* genotype combinations displayed significance on SBP or DBP in either the Canadian or Belgian hypertensives. Though, borderline significant models (SBP: $P=0.0558$; SBP: $P=0.0939$ respectively) were observed with the combination of the each of the CC-rs2288774 or AA-rs576416 genotypes with the GG-rs4149601 genotype in the Canadian males. The Canadian male population ($n=120$) is relatively small and this lack of power could have impeded observing a significant effect. The *NEDD4L* GG-rs4149601 and CC-rs2288774 interaction is previously described as having a positive effect on BP (Fava et al., 2006) and SS (Dahlberg et al., 2007). No significance was found with the rs288774 and rs576416 interaction, but a synergistic effect between rs4149601 and rs2288774 and rs576416 on age-related salt sensitive blood pressure.

Our study is one of the first attempting to investigate the genetic components of SS at the population level. The strength of this study was in the strict inclusion

criteria (section 6.1) and the higher level modeling using the interactions of not only important BP variables (age and sodium) but also multiple genetic regions. Even though measures were taken to control, and minimize potential confounders and misclassification of HTN our study still presented with some limitations. Beyond the methodological issue's previously discussed – lack of statistical power; non-linearity of DBP – there are some others that should be highlighted. Further population information surrounding other hypertension confounders such as the menopausal status of females, as well as smoking habits, would have benefited our statistical modeling analyses. Also, a lack of genotyping of rs2288774 in the Belgium population limited our ability to evaluate the previously reported synergism of rs4149601 and rs2288774 in a larger sample size. Additionally, this study was a cross-sectional snap-shot of our target hypertensive population. A prospective or longitudinal study design could have improved our ability to statistically evaluate the measured outcomes (Quinn, G. & Keough, 2002; Motulsky, 2014). Functionally speaking, little is still known about the *NEDD4L* rs4149601, rs2288774, and rs576416 SNPs. It seems well established that the A-rs4149601 variant causes a structural truncation of the NEDD4L protein lacking the C2 domain (Dunn et al., 2002), and research has shown that this could influence ENaC trafficking to and from the cellular membrane (Garrone et al., 2009). Beyond this, however, the literature lacks any substantial mechanical knowledge of this or the other two *NEDD4L* variations. Much of the research surrounding *NEDD4L* relies heavily on association studies, which are historically difficult to replicate unless prior knowledge exists that the SNP of interest affects a genes function (Colhoun,

McKeigue, & Smith, 2003). This idea suggests that much more work is needed at the molecular level surrounding these three *NEDD4L* SNPs before the genetic associations can be strongly considered.

Personalized genomic information has been proposed to impact pharmacological efficacy, among other things, and direct us towards the world of personalized medicine. With the continued decline of costs associated with genetic sequencing, even the discussion on using the human genome for assessing disease risk has been pushed to the forefront (Burke & Psaty, 2007; Guttmacher, McGuire, Ponder, & Stefánsson, 2010). An early example of evaluating genetic risk involves the BRCA1 and BRCA2 genes. BRCA 1/2, also known as tumour suppressor genes, are important in DNA repair and controlling cellular growth. BRCA 1/2 have been associated with 5-10% of breast (Campeau, Foulkes, & Tischkowitz, 2008) and near 15% of ovarian cancers (Pal et al., 2005). The benefits of identifying individuals at higher risk of developing specific cancers are obvious as patients can invest in a proactive approach towards prevention. For example, a bilateral prophylactic mastectomy in women with a mutation in BRCA 1/2 showed an almost 90% reduction in breast cancer risk (Rebbeck et al., 2004). There is clearly a positive reduction in cancer risk; however, the unique nature of cancer makes prevention challenging especially with regards to prophylactic treatments. The impact of removing at risk tissues can lead to other negative health outcomes highlighted by the psychological impact of mastectomies on body image (Gopie et al., 2013). In a population where approximately 70% of women report a change in satisfaction of

their body appearances post-mastectomy, it remains difficult to accept such risk reduction efforts as universally beneficial.

Chronic diseases, though, such as CVD and diabetes, are largely considered the result of poor behavioural practices (Prochaska, Wright, & Velicer, 2008). Utilizing genetic testing as a motivator for behavioural change could be an asset as maintaining positive behavioural change is notoriously challenging. (Panter-Brick, Clarke, Lomas, Pinder, & Lindsay, 2006). Grant et al. (2013) provide an example of a RCT, using personalized genetic risk counselling in order to motivate diabetes prevention behaviours. Their study underlines the challenges in promoting behavioural change, even with genetic risk counselling, as they did not find any significant behavioural changes for diabetes prevention in over-weight individuals. However, limitations, such as short study follow-up and intervention design could contribute to the lack of findings. Research has shown that successful behavioural interventions tend to involve: 1) the following of a theoretical framework; 2) building on local practices; 3) the targeting of receptive community members/stakeholders; 4) community mobilization; and, 5) the bolstering of individual knowledge and skills (Panter-Brick, Clarke, Lomas, Pinder, & Lindsay, 2006). Genetic screening for chronic illnesses should be considered a tool, one to be used to increase the personal knowledge of an individual in cooperation with a larger intervention strategy. Genetic screening, on it's own, is simply not a solution for population-wide prevention strategies (Guttmacher, McGuire, Ponder, & Stefánsson, 2010).

The investigation into the genetic susceptibility to the salt sensitive (SS) phenotype could positively influence future blood pressure reduction strategies. Beyond the obvious of more targeted dietary salt reductions, but the identification of genetic markers for SS could also reduce the costs, and expedite its classification. Current clinical identification practices involve comparing blood pressures from lengthy and costly dietary assessment protocols, requiring multiple weeks of adherence, on high, low, and normal sodium levels (Franco & Oparil, 2006; Overlack et al., 1993). With the identification of the genetic components involved with SS, it is perceivable that it could not only compliment population-based behavioural change strategies to reduce the burden of disease risk from HTN, but also improve individual care. Genetic testing, such as for *NEDD4L* loci, could be another tool for physicians to guide personal care, by providing more rapid SS HTN diagnoses and begin appropriate treatments for reducing blood pressure.

9.0 CONCLUSION

Developing the HTN phenotype is a complex process that can manifest from irregularities in multiple regulatory pathways. Identifying a model that accurately describes the variations in BP can be foreseeably elusive at a population level. This study was able to show that blood pressure was positively associated with increasing age in the Canadian and Belgian male hypertensive populations. Blood potassium had a strong negative association with BP in the Ottawa male population. The *NEDD4L* rs4149601 'G' allele was associated with an increase in SBP in hypertensive males from Belgium with increasing age and urine sodium levels. The 'GA' rs576416 *NEDD4L* genotype was also found to positively influence SBP in

Belgium male hypertensives. No interaction effects were identified between the three *NEDD4L* SNPs in either the Belgium or Canadian male hypertensive populations, though, a statistically non-significant trend was noticed in the Canadian male population, that the high-risk rs2288774 and rs576416 SNPs' alleles positively increased SBP when combined with the high-risk rs4149601 genotype. This study also highlighted the potential of sex effects on early-onset salt-sensitive hypertension.

10.0 BIBLIOGRAPHY

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[http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2288774]
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11.0 APPENDIX

11.1 Figures

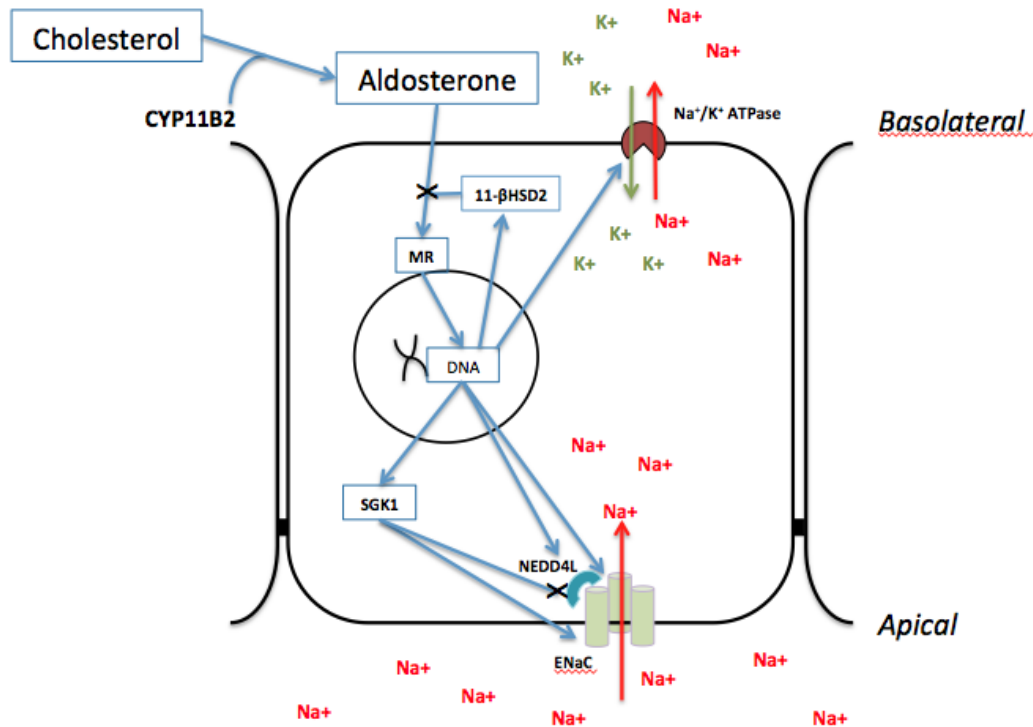


Figure 1: A simplified molecular overview of the signalling pathway in sodium reabsorption via the renin-angiotensin aldosterone system. The hormone, aldosterone, is converted from cholesterol in the *zona glomerulosa* of the adrenal cortex facilitated by aldosterone synthase (CYP11B2). Aldosterone then binds to the mineralocorticoid receptor (MR) creating a dimer, an active transcription factor. The MR stimulates the transcription of the machinery for sodium transport across epithelial cells: At the apical side of the epithelial cell the epithelial sodium channel (ENaC) creates a sodium specific channel from the lumen into the cellular space; At the basolateral surface a sodium/potassium pump (Na⁺/K⁺ ATPase) exchanges two extracellular potassium ions (K⁺) for three intracellular sodium (Na⁺) ions, completing the transfer of Na⁺ across the epithelial tissue; NEDD4L is a regulatory protein of the three ENaC subunits (α , β and γ) and their expression in the cellular membrane; SGK1 is an enzyme that inhibits the regulatory functions of NEDD4L through phosphorylation as well as directly stimulates ENaC expression; 11- β HSD2 is a protein that downregulates the effects of aldosterone through competitive inhibition of the MR.

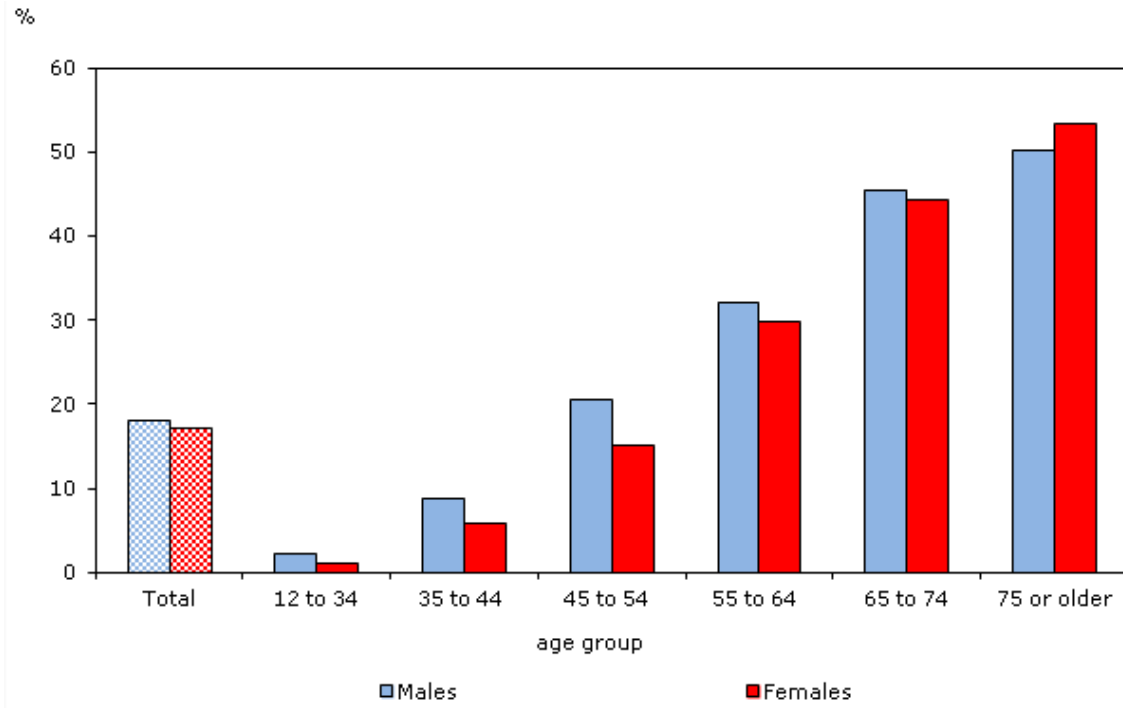


Figure 2: A graphical representation of the increasing trend of hypertension prevalence (shown in percentages) found in the Canadian male and female populations. Source: Stats Canada, 2013.

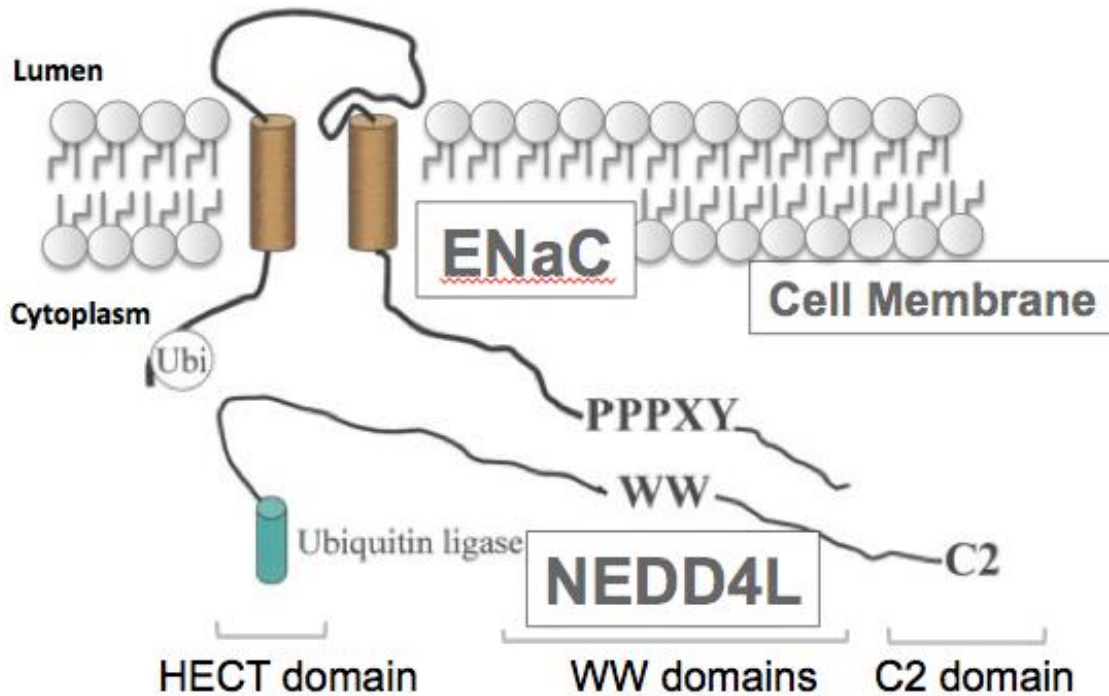


Figure 3: An illustration depicting the interaction of the NEDD4L protein with the epithelium sodium channel (ENaC). The NEDD4L protein has three distinct regions: (1) the C2 domain, is the Ca^{2+} sensitive region that responds to intracellular calcium levels; (2) two to four WW domains, the regions that interact with the intracellular PY motifs of the three ENaC - α , β or γ - subunits; and, (3) the HECT domain, an E3 enzymatic region responsible for tagging ENaC with ubiquitin for molecular recycling or proteasome degradation. Adapted from Stokes, 1999.



Figure 4: A linkage disequilibrium map of *NEDD4L* in Caucasian subjects from the Canadian and Polish cohorts. This plot highlights that the previously identified rs4149601 single nucleotide polymorphism is in high linkage disequilibrium (99) with rs576416, thus are commonly inherited together.

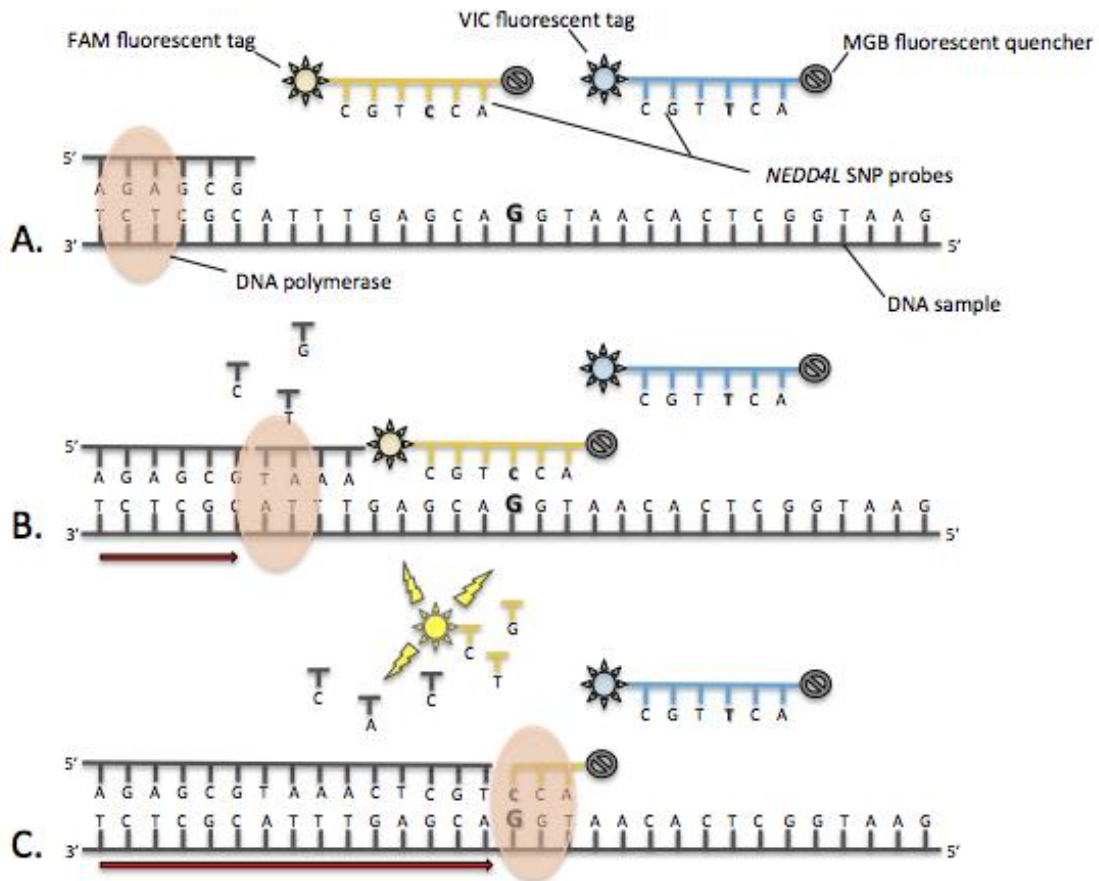
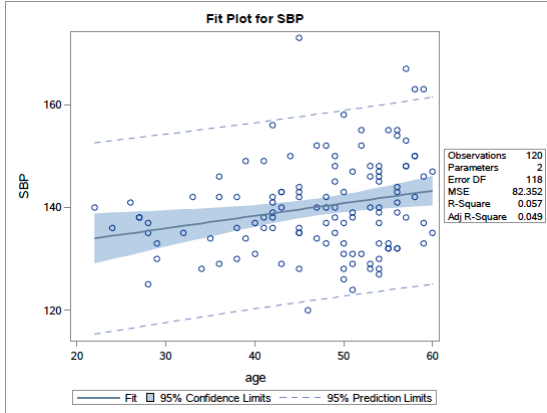
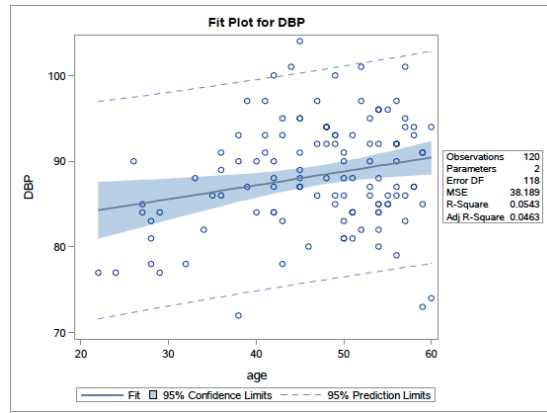


Figure 5: An illustration of the TaqMan MGB probe-based RT PCR process used in genotype identification of the *NEDD4L* rs4149601 and rs2288774 polymorphisms. (A) A solution of DNA with two *NEDD4L* probes, composed of the VIC and FAM identifying fluorescent tags and the associated MGB fluorescent quencher, for the targeted DNA region, along with DNA replication machinery (not shown). (B) Annealing, or association of the proper *NEDD4L* probe with the DNA sample during the replication process. (C) During the DNA replication process the fluorescent tag is cleaved from the probe, escaping the proximity of the MGB fluorescent quencher. This results in the emission of fluorescent light, which captured by the Bio-Rad CFX96 system (not shown) allowing for genotype identification.

Ottawa Males (n=120)

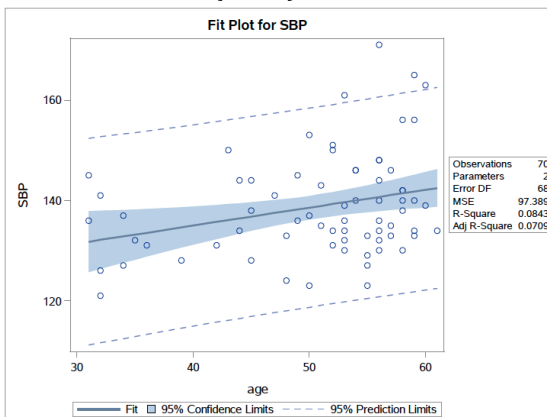


β	SE	P
0.244	0.078	0.002

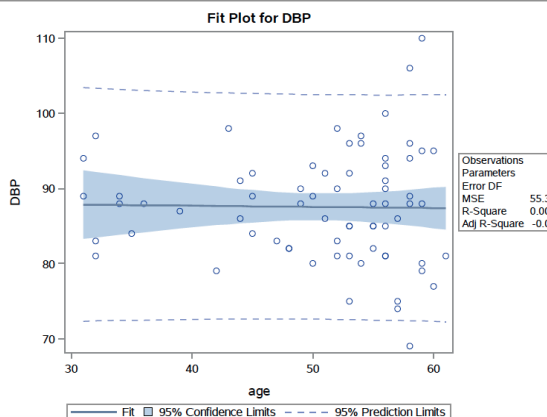


β	SE	P
0.162	0.064	0.0122

Ottawa Females (n=70)



β	SE	P
0.356	0.138	0.0118



β	SE	P
-0.015	0.104	NS

Figure 6: An example of the results of the simple linear regression of BP versus age in the Ottawa male (n=120) and female (n=70) hypertensive populations. The top two graphs demonstrate a positive relationship of age on both SBP (P=0.002) and DBP (P=0.012) in the Ottawa male hypertensives. The bottom two graphs demonstrate a positive association of SBP with age (P=0.012) in the Ottawa female hypertensives. No relationship was demonstrated in the Ottawa females for DBP (P>0.05). Complete simple regression results are found in Table 2.

11.2 Tables

Table 1: The demographic and clinical characteristics of the Canadian, Belgian and Polish hypertensive study populations, including populations' stratification by sex.

Variables	Total				Males				Females			
	Canada (n=190)	Belgium (n=377)	Poland (n=95)	P	Canada (n=120)	Belgium (n=273)	Poland (n=72)	P	Canada (n=70)	Belgium (n=104)	Poland (n=23)	P
Age, years	48±9	40±11	39±10	<0.001	47±9	39±11	39±11	<0.001	51±8	43±11	41±9	<0.001
Male sex, n (%)	120 (63.2)	273 (72.4)	72 (75.8)	0.033	-	-	-	-	-	-	-	-
BMI, kg/m ²	29.0±4.2	26.3±3.9	26.4±4.0	<0.001	29.4±4.0	26.5±3.8	27.2±3.5	<0.001	28.2±4.5	25.8±4.3	24.0±4.7	<0.001
SysBP (mmHg)	140±10	137±7	138±8	<0.001	140±9	137±7	138±7	0.001	139±10	136±7	137±10	0.079
DiaBP (mmHg)	88±7	85±8	89±7	<0.001	88±6	85±8	88±7	<0.001	88±7	86±6	90±7	0.021
Blood Na ⁺ (mmol/L)	140.0±2.0	141.7±3.3	140.4±2.3	<0.001	140.1±2.1	141.9±2.8	140.4±2.5	<0.001	139.8±1.8	141.1±4.4	140.1±1.8	0.048
Blood K ⁺ (mmol/L)	4.0±0.3	4.1±0.3	4.3±0.4	<0.001	4.0±0.3	4.1±0.3	4.4±0.5	<0.001	4.0±0.3	4.0±0.3	4.2±0.3	0.006
Corr. uNa ⁺ (mmol/day)	103±39	144±49	99±13	<0.001	94±31	142±47	104±9	<0.001	120±46	150±54	82±12	<0.001
Corr. uK ⁺ (mmol/day)	53.1±19.7	59.0±20.2	40.8±17.9	<0.001	47.7±14.5	56.0±19.3	43.7±18.9	<0.001	62.3±23.8	67.2±20.4	31.6±9.9	<0.001
Corr. uRatio (Na ⁺ /K ⁺)	2.12±0.92	2.67±1.44	2.84±1.22	<0.001	2.11±0.90	2.72±1.04	2.83±1.28	<0.001	2.14±0.97	2.52±2.19	2.86±1.02	0.159

P values were obtained by χ^2 test for categorical variables or ANOVA for continuous variables. Values are presented as n (%) and mean±SD.

Table 2: Results of a simple linear regression of blood pressure, systolic (SBP) and diastolic (DBP), versus age in the Canadian, Belgium, and Polish hypertensive study populations, which includes the populations' stratification for sex.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canada						
Males (n=120)	0.244	0.078	0.002	0.162	0.064	0.0122
Females (n=70)	0.356	0.138	0.0118	-0.015	0.104	NS
Belgium						
Males (n=273)	0.090	0.038	0.0191	0.310	0.041	<.0001
Females (n=104)	0.028	0.052	NS	0.009	0.058	NS
Poland						
Males (n=72)	0.120	0.079	0.1333	0.133	0.077	0.0874
Females (n=23)	-0.322	0.323	NS	-0.343	0.202	NS

Table 3: The results of a multiple linear regression using age, body mass index (BMI), blood sodium (Na⁺), blood potassium (K⁺), urine sodium corrected for urine creatinine (Corr.uNa⁺), urine potassium corrected for urine creatinine (Corr.uK⁺), and the ratio of Corr.uNa⁺ and Corr.uK⁺ (Corr.uRatio(Na⁺/K⁺)) versus systolic (SBP) and diastolic (DBP) blood pressure in the male hypertensive populations from Canada and Belgium.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.0682			0.0151
	R ² =0.1087			R ² =0.1409		
Age (yrs)	0.28325	0.09111	0.0024	0.17816	0.06831	0.0103
BMI (kg/m ²)	-0.25453	0.25579	NS	-0.2907	0.16753	0.0854
Blood Na ⁺ (mmol/L)	0.01337	0.40355	NS	-0.10808	0.29169	NS
Blood K ⁺ (mmol/L)	-3.78182	2.55037	0.1409	-3.77547	1.80791	0.039
Corr. uNa ⁺ (mmol/day)	-0.04663	0.08281	NS	0.05077	0.06998	NS
Corr. uK ⁺ (mmol/day)	0.02078	0.12567	NS	-0.05433	0.13686	NS
Corr. uRatio (Na ⁺ /K ⁺)	3.10677	3.70698	NS	-0.93213	2.81405	NS
Belgium Males (n=273)			0.0176			<.0001
	R ² =0.0624			R ² =0.2005		
Age (yrs)	0.03764	0.04458	NS	0.29136	0.04588	<.0001
BMI (kg/m ²)	0.37509	0.13085	0.0045	0.2057	0.12273	0.0949
Blood Na ⁺ (mmol/L)	0.09896	0.14955	NS	-0.10835	0.16833	NS
Blood K ⁺ (mmol/L)	0.18349	1.62458	NS	-0.09339	1.59782	NS
Corr. uNa ⁺ (mmol/day)	0.01008	0.01581	NS	-0.00558	0.01831	NS
Corr. uK ⁺ (mmol/day)	-0.00399	0.03635	NS	-0.02822	0.04236	NS
Corr. uRatio (Na ⁺ /K ⁺)	-0.88016	0.81379	NS	-0.05027	0.8519	NS

Table 4: The results of the backwards regression analyses in the male hypertensive populations' from Canada and Belgium. The final model includes both age and urine sodium corrected for urine creatinine (Corr.uNa+) if the variables were not found to be significant from the backward regression.

	SBP	DBP
Canadian Males (n=120)		
	Age (yrs) Blood K+ (mmol/L)	Age (yrs) BMI (kg/m ²) Blood K+ (mmol/L)
Final model:	Age (yrs) Blood K+ (mmol/L) Corr. uNa+ (mmol/day)	Age (yrs) BMI (kg/m ²) Blood K+ (mmol/L) Corr. uNa+ (mmol/day)
Belgium Males (n=273)		
	BMI (kg/m ²)	Age (yrs) BMI (kg/m ²)
Final model:	Age (yrs) BMI (kg/m ²) Corr. uNa+ (mmol/day)	Age (yrs) BMI (kg/m ²) Corr. uNa+ (mmol/day)

Table 5: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the rs414901 genotypes (GG, GA, AA) in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.1342			0.0200
	R ² =0.1442			R ² =0.2045		
(intercept)	132.96	33.28	0.0001	91.61	39.46	0.0222
Age (yrs)	0.67567	0.71386	NS	0.5127	0.79763	NS
BMI (kg/m ²)	-	-	-	-0.26256	0.15077	0.0845
Blood K ⁺ (mmol/L)	-4.63528	2.56307	0.0733	-4.77624	1.71235	0.0063
Corr. uNa ⁺ (mmol/day)	0.11292	0.36638	NS	0.06409	0.36576	NS
r4(GA)*Age*Corr.uNa ⁺	-0.00119	0.01083	NS	-0.00127	0.00993	NS
rs4(GG)*Age*Corr.uNa ⁺	0.00605	0.00825	NS	0.00471	0.00705	NS

Table 6: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa⁺) and the rs414901 genotypes (GG, GA, AA) in the Belgian male hypertensive population

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Belgium Males (n=273)			0.0034			<.0001
	R ² =0.1062			R ² =0.2262		
(intercept)	101.35	6.99	<0.0001	48.80	11.93	<0.0001
Age (yrs)	0.67082	0.1854	0.0004	0.81764	0.30246	0.0073
BMI (kg/m ²)	0.33212	0.12102	0.0065	0.2351	0.12211	0.0553
Corr. uNa ⁺ (mmol/day)	0.19946	0.04619	<0.0001	0.1326	0.07592	0.0819
rs4(GA)*Age*Corr.uNa ⁺	0.00369	0.00147	0.0129	0.00289	0.0022	0.1899
rs4(GG)*Age*Corr.uNa ⁺	0.00463	0.00174	0.0082	0.00328	0.00247	0.1854

Table 7: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the rs576416 genotypes (AA, GA, GG) in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.1545			0.0359
	R ² =0.1400			R ² =0.1904		
(intercept)	172.11	36.86	<0.0001	119.97	23.11	<0.0001
Age (yrs)	-0.23588	0.79824	NS	-0.18370	0.50818	NS
BMI (kg/m ²)	-	-	-	-0.25785	0.13661	0.0618
Blood K ⁺ (mmol/L)	-5.53878	2.77806	0.0487	-4.14963	1.76195	0.0204
Corr. uNa ⁺ (mmol/day)	-0.19555	0.40741	NS	-0.16077	0.23407	NS
rs5(GA)*Age*Corr.uNa ⁺	-0.01086	0.01016	NS	-0.00487	0.00635	NS
rs5(AA)*Age*Corr.uNa ⁺	0.01462	0.01286	NS	-0.00283	0.00828	NS

Table 8: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the rs576416 genotypes (AA, GA, GG) in the Belgian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Belgium Males (n=273)			0.0025			<.0001
	R ² =0.1092			R ² =0.2440		
(intercept)	109.20	6.07	<0.0001	55.33	6.28	<0.0001
Age (yrs)	0.50433	0.13727	0.0003	0.64560	0.15611	<0.0001
BMI (kg/m ²)	0.31622	0.12605	0.0127	0.22917	0.1272	0.0728
Corr. uNa ⁺ (mmol/day)	0.10990	0.03924	0.0055	0.07743	0.04026	0.0555
rs5(GA)*Age*Corr.uNa ⁺	0.00334	0.00134	0.0131	0.00305	0.00174	0.0811
rs5(AA)*Age*Corr.uNa ⁺	0.00241	0.00422	NS	0.00051969	0.00360	NS

Table 9: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the high risk rs4149601 (GG) and rs576416 (AA) genotypes in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.0558			0.0124
	R ² =0.1247			R ² =0.1694		
(intercept)	135.76	16.26	<0.0001	105.50	13.33	<0.0001
Age (yrs)	0.50652	0.37337	0.1777	0.04967	0.26722	NS
BMI (kg/m ²)	-	-	-	-0.25891	0.1434	0.0737
Blood K ⁺ (mmol/L)	-5.28176	2.62492	0.0466	-3.85909	1.67272	0.0229
Corr. uNa ⁺ (mmol/day)	0.15865	0.18712	NS	-0.00997	0.12674	NS
rs4(GG)* rs5(AA)*Age*Corr.uNa ⁺	0.02101	0.00993	0.0366	-0.00067	0.00648	NS

Table 10: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the high risk rs4149601 (GG) and rs576416 (AA) genotypes in the Belgian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Belgium Males (n=273)			0.0062			<.0001
	$R^2=0.0779$			$R^2=0.2279$		
(intercept)	120.64	5.05	<0.0001	64.63	6.10	<0.0001
Age (yrs)	0.19549	0.10886	0.0737	0.39062	0.13567	0.0043
BMI (kg/m ²)	0.32767	0.12863	0.0114	0.23519	0.12789	0.0670
Corr. uNa ⁺ (mmol/day)	0.04645	0.0279	0.0972	0.02912	0.03345	NS
rs4(GG)* rs5(AA)*Age*Corr.uNa ⁺	0.00081415	0.0042	NS	-0.00080925	0.00355	NS

Table 11: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the rs2288774 genotypes (CC, CT, TT) in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.2764			0.0504
	R ² =0.1207			R ² =0.1818		
(intercept)	160.43	55.68	0.0048	128.92	26.47	<0.0001
Age (yrs)	-0.02628	1.09989	NS	-0.48014	0.53659	NS
BMI (kg/m ²)	-	-	-	-0.30824	0.14625	0.0374
Blood K ⁺ (mmol/L)	-4.76249	2.74205	0.0853	-4.48189	1.93383	0.0224
Corr. uNa ⁺ (mmol/day)	-0.01701	0.5488	NS	-0.20033	0.23827	NS
rs2(CT)*Age*Corr.uNa ⁺	-0.0044	0.01199	NS	-0.00841	0.00626	0.1819
rs2(CC)*Age*Corr.uNa ⁺	0.00477	0.01459	NS	0.00437	0.00271	NS

Table 12: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the high risk rs4149601 (GG) and rs2288774 (CC) genotypes in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.0939			0.0355
	R ² =0.1122			R ² =0.1465		
(intercept)	137.29	16.28	<0.0001	104.08	13.37	<0.0001
Age (yrs)	0.37357	0.35449	NS	0.12117	0.23704	NS
BMI (kg/m ²)	-	-	-	-0.27427	0.15046	0.0710
Blood K ⁺ (mmol/L)	-4.04545	2.51358	0.1104	-4.04361	1.77461	0.0246
Corr. uNa ⁺ (mmol/day)	0.09853	0.17865	NS	0.00671	0.11104	NS
rs4(GG)*rs2(CC)*Age*Corr.uNa ⁺	0.02009	0.00974	0.0414	0.00794	0.02526	NS

Table 13: The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa+) and the high risk rs2288774 (CC) and rs576416 (AA) genotypes in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Canadian Males (n=120)			0.1141			0.0068
	$R^2=0.1073$			$R^2=0.1818$		
(intercept)	140.53	15.90	<0.0001	105.03	12.45	<0.0001
Age (yrs)	0.32048	0.3488	NS	0.12505	0.22777	NS
BMI (kg/m ²)	-	-	-	-0.26128	0.14557	0.0754
Blood K ⁺ (mmol/L)	-4.41617	2.53121	0.0838	-4.47066	1.6478	0.0077
Corr. uNa ⁺ (mmol/day)	0.07543	0.17687	NS	0.01082	0.10925	NS
rs2(CC)*rs5(AA)*Age*Corr.uNa ⁺	0.01853	0.14053	NS	0.01406	0.10312	NS

11.3 Supplementary

11.3.1 SAS 9.4.0 coding for regression analyses

```
/* Inputting the .csv file into SAS */
DATA sasuser.hyperdata;
    INFILE 'E:\Thesis\UPDATED 14.csv' dsd;
/*column titles */
    INPUT Location $ rs2 $ rs4 $ rs5 $ uC corr_uNA uNA corr_uK uK uRatio corr_uRatio bNA bK Gender BMI SBP DBP
age ;

/* Creating dummy variables for location */
    IF location = 'Ottawa' THEN loc_1 = 1; ELSE loc_1 = 0;
    IF location = 'Belgium' THEN loc_2 = 1; ELSE loc_2 = 0;
    IF location = 'Poland' THEN loc_3 = 1; ELSE loc_3 = 0;

/* Dummy Coding for each individual allele */

/* Creating dummy variables for rs2 */
    IF rs2 = 'CC' or rs2 = 'CT' THEN A_rs2_C = 1; ELSE A_rs2_C = 0;
    IF rs2 = 'CC' THEN A_rs2_CC = 1; ELSE A_rs2_CC = 0;
    IF rs2 = 'TT' or rs2 = 'CT' THEN A_rs2_T = 1; ELSE A_rs2_T = 0;
    IF rs2 = 'TT' THEN A_rs2_TT = 1; ELSE A_rs2_TT = 0;

/* Creating dummy variables for rs4 */
    IF rs4 = 'GG' or rs4 = 'GA' THEN A_rs4_G = 1; ELSE A_rs4_G = 0;
    IF rs4 = 'GG' THEN A_rs4_GG = 1; ELSE A_rs4_GG = 0;
    IF rs4 = 'AA' or rs4 = 'GA' THEN A_rs4_A = 1; ELSE A_rs4_A = 0;
    IF rs4 = 'AA' THEN A_rs4_AA = 1; ELSE A_rs4_AA = 0;

/* Creating dummy variables for rs5 */
    IF rs5 = 'GG' or rs5 = 'GA' THEN A_rs5_G = 1; ELSE A_rs5_G = 0;
    IF rs5 = 'GG' THEN A_rs5_GG = 1; ELSE A_rs5_GG = 0;
    IF rs5 = 'AA' or rs5 = 'GA' THEN A_rs5_A = 1; ELSE A_rs5_A = 0;
    IF rs5 = 'AA' THEN A_rs5_AA = 1; ELSE A_rs5_AA = 0;

/* Dummy Coding for each individual Genotype */

/* Creating dummy variables for rs2 */
    IF rs2 = 'CC' THEN G_rs2_CC = 1; ELSE G_rs2_CC = 0;
    IF rs2 = 'CT' THEN G_rs2_CT = 1; ELSE G_rs2_CT = 0;
/* Creating dummy variables for rs4 */
    IF rs4 = 'GG' THEN G_rs4_GG = 1; ELSE G_rs4_GG = 0;
    IF rs4 = 'GA' THEN G_rs4_GA = 1; ELSE G_rs4_GA = 0;
/* Creating dummy variables for rs5 */
    IF rs5 = 'GG' THEN G_rs5_GG = 1; ELSE G_rs5_GG = 0;
    IF rs5 = 'GA' THEN G_rs5_GA = 1; ELSE G_rs5_GA = 0;
    IF rs5 = 'AA' THEN G_rs5_AA = 1; ELSE G_rs5_AA = 0;

RUN;

/* Creating the interaction terms */

DATA sasuser.hyperdataTERMS;
    set sasuser.hyperdata;

    age_uNa = age*uNa ;
    age_CORRuNa = age*corr_una ;

    OTT_A_rs4_G = A_rs4_G*loc_1;
    BELG_A_rs4_G = A_rs4_G*loc_2;
    POL_A_rs4_G = A_rs4_G*loc_3;

    OTT_A_rs4_GG = A_rs4_GG*loc_1;
    BELG_A_rs4_GG = A_rs4_GG*loc_2;
    POL_A_rs4_GG = A_rs4_GG*loc_3;

    OTT_A_rs2_C = A_rs2_C*loc_1;
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BELG_A_rs2_C = A_rs2_C*loc_2;
POL_A_rs2_C = A_rs2_C*loc_3;

OTT_A_rs2_CC = A_rs2_CC*loc_1;
BELG_A_rs2_CC = A_rs2_CC*loc_2;
POL_A_rs2_CC = A_rs2_CC*loc_3;

OTT_A_rs5_A = A_rs5_A*loc_1;
BELG_A_rs5_A = A_rs5_A*loc_2;
POL_A_rs5_A = A_rs5_A*loc_3;

OTT_A_rs5_AA = A_rs5_AA*loc_1;
BELG_A_rs5_AA = A_rs5_AA*loc_2;
POL_A_rs5_AA = A_rs5_AA*loc_3;

OTT_A_rs4_A = A_rs4_A*loc_1;
BELG_A_rs4_A = A_rs4_A*loc_2;
POL_A_rs4_A = A_rs4_A*loc_3;

OTT_A_rs4_AA = A_rs4_AA*loc_1;
BELG_A_rs4_AA = A_rs4_AA*loc_2;
POL_A_rs4_AA = A_rs4_AA*loc_3;

OTT_A_rs2_T = A_rs2_T*loc_1;
BELG_A_rs2_T = A_rs2_T*loc_2;
POL_A_rs2_T = A_rs2_T*loc_3;

OTT_A_rs2_TT = A_rs2_TT*loc_1;
BELG_A_rs2_TT = A_rs2_TT*loc_2;
POL_A_rs2_TT = A_rs2_TT*loc_3;

OTT_A_rs5_G = A_rs5_G*loc_1;
BELG_A_rs5_G = A_rs5_G*loc_2;
POL_A_rs5_G = A_rs5_G*loc_3;

OTT_A_rs5_GG = A_rs5_GG*loc_1;
BELG_A_rs5_GG = A_rs5_GG*loc_2;
POL_A_rs5_GG = A_rs5_GG*loc_3;

/* Age*allele */
A_rs4_age_G = A_rs4_G*age;
A_rs4_age_GG = A_rs4_GG*age;
A_rs4_age_A = A_rs4_A*age;
A_rs4_age_AA = A_rs4_AA*age;
A_rs2_age_C = A_rs2_C*age;
A_rs2_age_CC = A_rs2_CC*age;
A_rs2_age_T = A_rs2_T*age;
A_rs2_age_TT = A_rs2_TT*age;
A_rs5_age_G = A_rs5_G*age;
A_rs5_age_GG = A_rs5_GG*age;
A_rs5_age_A = A_rs5_A*age;
A_rs5_age_AA = A_rs5_AA*age;

/* uNa*allele */
A_rs4_uNa_G = A_rs4_G*uNa;
A_rs4_uNa_GG = A_rs4_GG*uNa;
A_rs2_uNa_C = A_rs2_C*uNa;
A_rs2_uNa_CC = A_rs2_CC*uNa;
A_rs5_uNa_G = A_rs5_G*uNa;
A_rs5_uNa_GG = A_rs5_GG*uNa;

/* CORR uNa*allele */
A_rs4_CORR_uNa_G = A_rs4_G*corr_una;
A_rs4_CORR_uNa_GG = A_rs4_GG*corr_una;
A_rs2_CORR_uNa_C = A_rs2_C*corr_una;
A_rs2_CORR_uNa_CC = A_rs2_CC*corr_una;
A_rs5_CORR_uNa_G = A_rs5_G*corr_una;
A_rs5_CORR_uNa_GG = A_rs5_GG*corr_una;
A_rs5_CORR_uNa_A = A_rs5_A*corr_una;

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A_rs5_CORR_uNa_AA = A_rs5_AA*corr_una;

/* Age*uNa*allele */
A_rs4_age_uNa_G = A_rs4_G*age_uNa;
A_rs4_age_uNa_GG = A_rs4_GG*age_uNa;
A_rs2_age_uNa_C = A_rs2_C*age_uNa;
A_rs2_age_uNa_CC = A_rs2_CC*age_uNa;
A_rs5_age_uNa_G = A_rs5_G*age_uNa;
A_rs5_age_uNa_GG = A_rs5_GG*age_uNa;

/* Age*CORRuNa*allele */

A_rs4_age_CORRuNa_G = A_rs4_G*age_corrNa;
A_rs4_age_CORRuNa_GG = A_rs4_GG*age_corrNa;
A_rs2_age_CORRuNa_C = A_rs2_C*age_corrNa;
A_rs2_age_CORRuNa_CC = A_rs2_CC*age_corrNa;
A_rs5_age_CORRuNa_G = A_rs5_G*age_corrNa;
A_rs5_age_CORRuNa_GG = A_rs5_GG*age_corrNa;
A_rs5_age_CORRuNa_A = A_rs5_A*age_corrNa;
A_rs5_age_CORRuNa_AA = A_rs5_AA*age_corrNa;

/* Age_corrNa_allele by location */
OTT_A_rs4_age_CORRuNa_G = A_rs4_age_CORRuNa_G*loc_1;
OTT_A_rs4_age_CORRuNa_GG = A_rs4_age_CORRuNa_GG*loc_1;
OTT_A_rs2_age_CORRuNa_C = A_rs2_age_CORRuNa_C*loc_1;
OTT_A_rs2_age_CORRuNa_CC = A_rs2_age_CORRuNa_CC*loc_1;
OTT_A_rs5_age_CORRuNa_G = A_rs5_age_CORRuNa_G*loc_1;
OTT_A_rs5_age_CORRuNa_GG = A_rs5_age_CORRuNa_GG*loc_1;
OTT_A_rs5_age_CORRuNa_A = A_rs5_age_CORRuNa_A*loc_1;
OTT_A_rs5_age_CORRuNa_AA = A_rs5_age_CORRuNa_AA*loc_1;

BELG_A_rs4_age_CORRuNa_G = A_rs4_age_CORRuNa_G*loc_2;
BELG_A_rs4_age_CORRuNa_GG = A_rs4_age_CORRuNa_GG*loc_2;
BELG_A_rs2_age_CORRuNa_C = A_rs2_age_CORRuNa_C*loc_2;
BELG_A_rs2_age_CORRuNa_CC = A_rs2_age_CORRuNa_CC*loc_2;
BELG_A_rs5_age_CORRuNa_G = A_rs5_age_CORRuNa_G*loc_2;
BELG_A_rs5_age_CORRuNa_GG = A_rs5_age_CORRuNa_GG*loc_2;
BELG_A_rs5_age_CORRuNa_A = A_rs5_age_CORRuNa_A*loc_2;
BELG_A_rs5_age_CORRuNa_AA = A_rs5_age_CORRuNa_AA*loc_2;

POL_A_rs4_age_CORRuNa_G = A_rs4_age_CORRuNa_G*loc_3;
POL_A_rs4_age_CORRuNa_GG = A_rs4_age_CORRuNa_GG*loc_3;
POL_A_rs2_age_CORRuNa_C = A_rs2_age_CORRuNa_C*loc_3;
POL_A_rs2_age_CORRuNa_CC = A_rs2_age_CORRuNa_CC*loc_3;
POL_A_rs5_age_CORRuNa_G = A_rs5_age_CORRuNa_G*loc_3;
POL_A_rs5_age_CORRuNa_GG = A_rs5_age_CORRuNa_GG*loc_3;
POL_A_rs5_age_CORRuNa_A = A_rs5_age_CORRuNa_A*loc_3;
POL_A_rs5_age_CORRuNa_AA = A_rs5_age_CORRuNa_AA*loc_3;

/* Age*genotype */
G_rs4_age_GG = G_rs4_GG*age;
G_rs4_age_GA = G_rs4_GA*age;
G_rs2_age_CC = G_rs2_CC*age;
G_rs2_age_CT = G_rs2_CT*age;
G_rs5_age_GG = G_rs5_GG*age;
G_rs5_age_GA = G_rs5_GA*age;
G_rs5_age_AA = G_rs5_AA*age;

/* uNa*genotype */
G_rs4_uNa_GG = G_rs4_GG*uNa;
G_rs4_uNa_GA = G_rs4_GA*uNa;
G_rs2_uNa_CC = G_rs2_CC*uNa;
G_rs2_uNa_CT = G_rs2_CT*uNa;
G_rs5_uNa_GG = G_rs5_GG*uNa;
G_rs5_uNa_GA = G_rs5_GA*uNa;

/* CORRuNa*genotype */
G_rs4_CORR_uNa_GG = G_rs4_GG*corr_una;
G_rs4_CORR_uNa_GA = G_rs4_GA*corr_una;
G_rs2_CORR_uNa_CC = G_rs2_CC*corr_una;

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G_rs2_CORR_uNa_CT = G_rs2_CT*corr_una;
G_rs5_CORR_uNa_GG = G_rs5_GG*corr_una;
G_rs5_CORR_uNa_GA = G_rs5_GA*corr_una;
G_rs5_CORR_uNa_AA = G_rs5_AA*corr_una;
/* Age*uNa*genotype */
G_rs4_age_uNa_GG = G_rs4_GG*age_uNa;
G_rs4_age_uNa_GA = G_rs4_GA*age_uNa;
G_rs2_age_uNa_CC = G_rs2_CC*age_uNa;
G_rs2_age_uNa_CT = G_rs2_CT*age_uNa;
G_rs5_age_uNa_GG = G_rs5_GG*age_uNa;
G_rs5_age_uNa_GA = G_rs5_GA*age_uNa;
/* Age*CORRuNa*genotype */
G_rs4_age_CORRuNa_GG = G_rs4_GG*age_corr_uNa;
G_rs4_age_CORRuNa_GA = G_rs4_GA*age_corr_uNa;
G_rs2_age_CORRuNa_CC = G_rs2_CC*age_corr_uNa;
G_rs2_age_CORRuNa_CT = G_rs2_CT*age_corr_uNa;
G_rs5_age_CORRuNa_GG = G_rs5_GG*age_corr_uNa;
G_rs5_age_CORRuNa_GA = G_rs5_GA*age_corr_uNa;
G_rs5_age_CORRuNA_AA = G_rs5_AA*age_corr_uNa;

/* Allele: rs4*rs2 ; rs4*rs5 */
A_rs4_rs2_G_C = A_rs4_G*A_rs2_C;
A_rs4_rs2_G_CC = A_rs4_G*A_rs2_CC;
A_rs4_rs2_GG_C = A_rs4_GG*A_rs2_C;
A_rs4_rs2_GG_CC = A_rs4_GG*A_rs2_CC;

A_rs4_rs5_G_G = A_rs4_G*A_rs5_G;
A_rs4_rs5_G_GG = A_rs4_G*A_rs5_GG;
A_rs4_rs5_GG_G = A_rs4_GG*A_rs5_G;
A_rs4_rs5_GG_GG = A_rs4_GG*A_rs5_GG;
A_rs4_rs5_G_A = A_rs4_G*A_rs5_A;
A_rs4_rs5_G_AA = A_rs4_G*A_rs5_AA;
A_rs4_rs5_GG_A = A_rs4_GG*A_rs5_A;
A_rs4_rs5_GG_AA = A_rs4_GG*A_rs5_AA;

/* Allele: rs4rs2 ; rs4rs5* age */
A_rs4_rs2_G_C_AGE = A_rs4_rs2_G_C*age;
A_rs4_rs2_G_CC_AGE = A_rs4_rs2_G_CC*age;
A_rs4_rs2_GG_C_AGE = A_rs4_rs2_GG_C*age;
A_rs4_rs2_GG_CC_AGE = A_rs4_rs2_GG_CC*age;

A_rs4_rs5_G_G_AGE = A_rs4_rs5_G_G*age;
A_rs4_rs5_G_GG_AGE = A_rs4_rs5_G_GG*age;
A_rs4_rs5_GG_G_AGE = A_rs4_rs5_GG_G*age;
A_rs4_rs5_GG_GG_AGE = A_rs4_rs5_GG_GG*age;
A_rs4_rs5_G_A_AGE = A_rs4_rs5_G_A*age;
A_rs4_rs5_G_AA_AGE = A_rs4_rs5_G_AA*age;
A_rs4_rs5_GG_A_AGE = A_rs4_rs5_GG_A*age;
A_rs4_rs5_GG_AA_AGE = A_rs4_rs5_GG_AA*age;

/* Allele: rs4rs2 ; rs4rs5* uNa */
A_rs4_rs2_G_C_UNA = A_rs4_rs2_G_C*uNa;
A_rs4_rs2_G_CC_UNA = A_rs4_rs2_G_CC*uNa;
A_rs4_rs2_GG_C_UNA = A_rs4_rs2_GG_C*uNa;
A_rs4_rs2_GG_CC_UNA = A_rs4_rs2_GG_CC*uNa;

A_rs4_rs5_G_G_UNA = A_rs4_rs5_G_G*uNa;
A_rs4_rs5_G_GG_UNA = A_rs4_rs5_G_GG*uNa;
A_rs4_rs5_GG_G_UNA = A_rs4_rs5_GG_G*uNa;
A_rs4_rs5_GG_GG_UNA = A_rs4_rs5_GG_GG*uNa;

/* Allele: rs4rs2 ; rs4rs5* CORR_uNa */
A_rs4_rs2_G_C_corr_UNA = A_rs4_rs2_G_C*corr_uNa;
A_rs4_rs2_G_CC_corr_UNA = A_rs4_rs2_G_CC*corr_uNa;
A_rs4_rs2_GG_C_corr_UNA = A_rs4_rs2_GG_C*corr_uNa;
A_rs4_rs2_GG_CC_corr_UNA = A_rs4_rs2_GG_CC*corr_uNa;

A_rs4_rs5_G_G_corr_UNA = A_rs4_rs5_G_G*corr_uNa;
A_rs4_rs5_G_GG_corr_UNA = A_rs4_rs5_G_GG*corr_uNa;

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A_rs4_rs5_GG_G_corr_UNA = A_rs4_rs5_GG_G*corr_uNa;
A_rs4_rs5_GG_GG_corr_UNA = A_rs4_rs5_GG_GG*corr_uNa;
A_rs4_rs5_G_A_corr_UNA = A_rs4_rs5_G_A*corr_uNa;
A_rs4_rs5_G_AA_corr_UNA = A_rs4_rs5_G_AA*corr_uNa;
A_rs4_rs5_GG_A_corr_UNA = A_rs4_rs5_GG_A*corr_uNa;
A_rs4_rs5_GG_AA_corr_UNA = A_rs4_rs5_GG_AA*corr_uNa;

/* Allele: rs4rs2 ; rs4rs5* Age*uNa */
A_rs4_rs2_G_C_AGEUNA = A_rs4_rs2_G_C*age_uNa;
A_rs4_rs2_G_CC_AGEUNA = A_rs4_rs2_G_CC*age_uNa;
A_rs4_rs2_GG_C_AGEUNA = A_rs4_rs2_GG_C*age_uNa;
A_rs4_rs2_GG_CC_AGEUNA = A_rs4_rs2_GG_CC*age_uNa;

A_rs4_rs5_G_G_AGEUNA = A_rs4_rs5_G_G*age_uNa;
A_rs4_rs5_G_GG_AGEUNA = A_rs4_rs5_G_GG*age_uNa;
A_rs4_rs5_GG_G_AGEUNA = A_rs4_rs5_GG_G*age_uNa;
A_rs4_rs5_GG_GG_AGEUNA = A_rs4_rs5_GG_GG*age_uNa;

/* Allele: rs4rs2 ; rs4rs5* Age*corr_uNa */
A_rs4_rs2_G_C_AGEcorrUNA = A_rs4_rs2_G_C*age_corr_uNa;
A_rs4_rs2_G_CC_AGEcorrUNA = A_rs4_rs2_G_CC*age_corr_uNa;
A_rs4_rs2_GG_C_AGEcorrUNA = A_rs4_rs2_GG_C*age_corr_uNa;
A_rs4_rs2_GG_CC_AGEcorrUNA = A_rs4_rs2_GG_CC*age_corr_uNa;

A_rs4_rs5_G_G_AGEcorrUNA = A_rs4_rs5_G_G*age_corr_uNa;
A_rs4_rs5_G_GG_AGEcorrUNA = A_rs4_rs5_G_GG*age_corr_uNa;
A_rs4_rs5_GG_G_AGEcorrUNA = A_rs4_rs5_GG_G*age_corr_uNa;
A_rs4_rs5_GG_GG_AGEcorrUNA = A_rs4_rs5_GG_GG*age_corr_uNa;

A_rs4_rs5_G_A_AGEcorrUNA = A_rs4_rs5_G_A*age_corr_uNa;
A_rs4_rs5_G_AA_AGEcorrUNA = A_rs4_rs5_G_AA*age_corr_uNa;
A_rs4_rs5_GG_A_AGEcorrUNA = A_rs4_rs5_GG_A*age_corr_uNa;
A_rs4_rs5_GG_AA_AGEcorrUNA = A_rs4_rs5_GG_AA*age_corr_uNa;

/* Genotype: rs4*rs2 ; rs4*rs5 */
G_rs4_rs2_GGCC = G_rs4_GG*G_rs2_CC;
G_rs4_rs2_GGCT = G_rs4_GG*G_rs2_CT;
G_rs4_rs2_GACC = G_rs4_GA*G_rs2_CC;
G_rs4_rs2_GACT = G_rs4_GA*G_rs2_CT;

G_rs4_rs5_GGGG = G_rs4_GG*G_rs5_GG;
G_rs4_rs5_GGGA = G_rs4_GG*G_rs5_GA;
G_rs4_rs5_GGAA = G_rs4_GG*G_rs5_AA;
G_rs4_rs5_GAGG = G_rs4_GA*G_rs5_GG;
G_rs4_rs5_GAGA = G_rs4_GA*G_rs5_GA;

G_rs2_rs5_CCAA = G_rs2_CC*G_rs5_AA;
G_rs2_rs5_CCGG = G_rs2_CC*G_rs5_GG;

/* Genotype: rs4rs2 ; rs4rs5* age */
G_rs4_rs2_GGCC_AGE = G_rs4_rs2_GGCC*age;
G_rs4_rs2_GGCT_AGE = G_rs4_rs2_GGCT*age;
G_rs4_rs2_GACC_AGE = G_rs4_rs2_GACC*age;
G_rs4_rs2_GACT_AGE = G_rs4_rs2_GACT*age;

G_rs4_rs5_GGGG_AGE = G_rs4_rs5_GGGG*age;
G_rs4_rs5_GGGA_AGE = G_rs4_rs5_GGGA*age;
G_rs4_rs5_GGAA_AGE = G_rs4_rs5_GGAA*age;
G_rs4_rs5_GAGG_AGE = G_rs4_rs5_GAGG*age;
G_rs4_rs5_GAGA_AGE = G_rs4_rs5_GAGA*age;

G_rs2_rs5_CCAA_AGE = G_rs2_rs5_CCAA*age;
G_rs2_rs5_CCGG_AGE = G_rs2_rs5_CCGG*age;

/* Genotype: rs4rs2 ; rs4rs5* uNa */
G_rs4_rs2_GGCC_UNA = G_rs4_rs2_GGCC*uNa;
G_rs4_rs2_GGCT_UNA = G_rs4_rs2_GGCT*uNa;
G_rs4_rs2_GACC_UNA = G_rs4_rs2_GACC*uNa;

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G_rs4_rs2_GACT_UNA = G_rs4_rs2_GACT*uNa;

G_rs4_rs5_GGGG_UNA = G_rs4_rs5_GGGG*uNa;
G_rs4_rs5_GGGA_UNA = G_rs4_rs5_GGGA*uNa;
G_rs4_rs5_GAGG_UNA = G_rs4_rs5_GAGG*uNa;
G_rs4_rs5_GAGA_UNA = G_rs4_rs5_GAGA*uNa;

/* Genotype: rs4rs2 ; rs4rs5* CORR_uNa */
G_rs4_rs2_GGCC_corr_UNA = G_rs4_rs2_GGCC*corr_uNa;
G_rs4_rs2_GGCT_corr_UNA = G_rs4_rs2_GGCT*corr_uNa;
G_rs4_rs2_GACC_corr_UNA = G_rs4_rs2_GACC*corr_uNa;
G_rs4_rs2_GACT_corr_UNA = G_rs4_rs2_GACT*corr_uNa;

G_rs4_rs5_GGGG_corr_UNA = G_rs4_rs5_GGGG*corr_uNa;
G_rs4_rs5_GGGA_corr_UNA = G_rs4_rs5_GGGA*corr_uNa;
G_rs4_rs5_GGAA_corr_UNA = G_rs4_rs5_GGAA*corr_uNa;
G_rs4_rs5_GAGG_corr_UNA = G_rs4_rs5_GAGG*corr_uNa;
G_rs4_rs5_GAGA_corr_UNA = G_rs4_rs5_GAGA*corr_uNa;

G_rs2_rs5_CCAA_corr_UNA = G_rs2_rs5_CCAA*corr_uNa;
G_rs2_rs5_CCGG_corr_UNA = G_rs2_rs5_CCGG*corr_uNa;

/* Genotype: rs4rs2 ; rs4rs5* Age*uNa */
G_rs4_rs2_GGCC_AGEUNA = G_rs4_rs2_GGCC*age_uNa;
G_rs4_rs2_GGCT_AGEUNA = G_rs4_rs2_GGCT*age_uNa;
G_rs4_rs2_GACC_AGEUNA = G_rs4_rs2_GACC*age_uNa;
G_rs4_rs2_GACT_AGEUNA = G_rs4_rs2_GACT*age_uNa;

G_rs4_rs5_GGGG_AGEUNA = G_rs4_rs5_GGGG*age_uNa;
G_rs4_rs5_GGGA_AGEUNA = G_rs4_rs5_GGGA*age_uNa;
G_rs4_rs5_GAGG_AGEUNA = G_rs4_rs5_GAGG*age_uNa;
G_rs4_rs5_GAGA_AGEUNA = G_rs4_rs5_GAGA*age_uNa;

/* Genotype: rs4rs2 ; rs4rs5* Age*corr_uNa */
G_rs4_rs2_GGCC_AGEcorrUNA = G_rs4_rs2_GGCC*age_corr_uNa;
G_rs4_rs2_GGCT_AGEcorrUNA = G_rs4_rs2_GGCT*age_corr_uNa;
G_rs4_rs2_GACC_AGEcorrUNA = G_rs4_rs2_GACC*age_corr_uNa;
G_rs4_rs2_GACT_AGEcorrUNA = G_rs4_rs2_GACT*age_corr_uNa;

G_rs4_rs5_GGGG_AGEcorrUNA = G_rs4_rs5_GGGG*age_corr_uNa;
G_rs4_rs5_GGGA_AGEcorrUNA = G_rs4_rs5_GGGA*age_corr_uNa;
G_rs4_rs5_GGAA_AGEcorrUNA = G_rs4_rs5_GGAA*age_corr_uNa;
G_rs4_rs5_GAGG_AGEcorrUNA = G_rs4_rs5_GAGG*age_corr_uNa;
G_rs4_rs5_GAGA_AGEcorrUNA = G_rs4_rs5_GAGA*age_corr_uNa;

G_rs2_rs5_CCAA_AGEcorrUNA = G_rs2_rs5_CCAA*age_corr_uNa;
G_rs2_rs5_CCGG_AGEcorrUNA = G_rs2_rs5_CCGG*age_corr_uNa;

RUN;

/* ----- */

/* Pay close attention to the terms used in the regressions as well as the 'location' and 'gender' */

/* ----- */

/* simple regression with age (ex. Ottawa males using SBP) */

ODS PDF;
ODS graphics on;

PROC REG data=sasuser.HyperdataTERMS plots=(qqplot diagnostics) ;
    where Location = "Ottawa" and Gender = 0;
    MODEL SBP = age / acov ACOVMETHOD=3 vif collin;

RUN;

ODS graphics off;
ODS PDF close;

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/* ----- */

/* Backwards regression For each population (ex. Ottawa using SBP) */

ODS PDF;
ODS graphics on;

PROC REG data=sasuser.HyperdataTERMS plots=(qqplot diagnostics);
      where Location = "Ottawa" and Gender = 0;
      MODEL SBP = age gender BMI bNa bK corr_uNa corr_uK corr_uRatio / acov ACOVMETHOD=3 vif collin SELECTION
= BACKWARD;

RUN;

ODS graphics off;
ODS PDF close;

/* ----- */

/* ULTIMATE Hypothesis Q for each population - single allele (ex. Ottawa males using SBP and rs4) */

ODS PDF;
ODS graphics on;

PROC REG data=sasuser.HyperdataTERMS plots=(qqplot diagnostics);
      where Location = "Ottawa" and Gender = 0;
      MODEL SBP = age bk corr_uNa age_CORRuNa A_rs4_G A_rs4_GG A_rs4_age_G A_rs4_age_GG A_rs4_CORRuNa_G
A_rs4_CORRuNa_GG A_rs4_age_CORRuNa_G A_rs4_age_CORRuNa_GG / acov ACOVMETHOD=3 vif collin;

RUN;

ODS graphics off;
ODS PDF close;

/* ----- */

/* Hypothesis Q for each population - single genotype (ex. Ottawa males using SBP and rs4) */

ODS PDF;
ODS graphics on;

PROC REG data=sasuser.HyperdataTERMS plots=(qqplot diagnostics);
      where Location = "Ottawa" and Gender = 0;
      MODEL SBP = age bk corr_uNa age_CORRuNa G_rs4_GG G_rs4_GA G_rs4_age_GG G_rs4_age_GA G_rs4_CORRuNa_GG
G_rs4_CORRuNa_GA G_rs4_age_CORRuNa_GG G_rs4_age_CORRuNa_GA / acov ACOVMETHOD=3 vif collin;

RUN;

ODS graphics off;
ODS PDF close;

/* ----- */

/* Hypothesis Q for each population - two genotypes [high risk genotypes - rs4(GG)/rs2(CC)/rs5(AA)] (ex. Ottawa males
using SBP and rs2 and rs5) */

ODS PDF;
ODS graphics on;

PROC REG data=sasuser.HyperdataTERMS plots=(qqplot diagnostics);
      where Location = "Ottawa" and Gender = 0;
      MODEL SBP = age bk corr_uNa age_CORRuNa G_rs4_rs2_GGCC G_rs4_rs2_GGCC_AGE G_rs4_rs2_GGCC_corr_UNA
G_rs4_rs2_GGCC_AGEcorrUNA / acov ACOVMETHOD=3 vif collin;

RUN;

ODS graphics off;
ODS PDF close;

```

11.3.2 Supplementary table

Supplementary Table 1 The results of the multiple regression analysis using the variables identified from the backwards regression variables including the interaction between age, corrected urine sodium (Corr.uNa⁺) and the rs576416 genotypes (AA, GA, GG) in the Canadian male hypertensive population.

Variables	SBP			DBP		
	β	SE	P	β	SE	P
Belgium Males (n=273)			0.0025			<.0001
	R ² =0.1092			R ² =0.2440		
(intercept)	113.42302	23.05672	<.0001	47.82851	21.45069	0.0266
Age (yrs)	0.37235	0.66099	NS	0.89526	0.63420	0.1593
BMI (kg/m ²)	0.31622	0.12605	0.0127	0.22917	0.12720	0.0728
Corr. uNa ⁺ (mmol/day)	0.01698	0.15304	NS	0.04941	0.10666	NS
rs5(GA)*Age*Corr.uNa ⁺	0.00093133	0.00424	NS	0.00253	0.00374	NS
rs5(GG)*Age*Corr.uNa ⁺	-0.00334	0.00134	0.0131	-0.00051969	0.00360	NS

11.3.3 Manuscript under review to the *Journal of Hypertension* for publication

Manuscript number: JH-D-16-00202

Status: Under Review

Journal of Hypertension

Association between the NEDD4L rs4149601 single nucleotide polymorphism and essential hypertension: a systematic review and meta-analysis

--Manuscript Draft--

Manuscript Number:	
Article Type:	De Novo Submission
Keywords:	Essential hypertension; NEDD4L; rs4149601; genetic association study; meta-analysis
Corresponding Author:	Frédérique Tesson, Ph.D. University of Ottawa Ottawa, ON CANADA
First Author:	Linlu Zhao
Order of Authors:	Linlu Zhao Suzan Chen Stephen Kutcher Hannah Nicolas Frans Leenen Frédérique Tesson, Ph.D.
Abstract:	<p>Objective: A loss-of-function variation (rs4149601) in the ubiquitin protein ligase NEDD4-Like (NEDD4L) gene has been implicated as a risk factor for essential hypertension. However, this finding has been inconsistently replicated. A systematic review and meta-analysis was conducted to assess and synthesize available evidence on the association between the NEDD4L rs4149601 single nucleotide polymorphism (SNP) and essential hypertension.</p> <p>Methods: This study conformed to PRISMA guidelines. Four databases were systematically searched following a sensitive strategy to identify relevant studies for inclusion in the systematic review and meta-analysis. The inverse variance method was used to calculate summary odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for the allelic and genotypic comparisons.</p> <p>Results: Seventy-two non-duplicative studies were identified from database and hand searches and screened for eligibility. Meta-analysis of seven studies found a non-significant overall association between rs4149601 and essential hypertension. However, subgroup analysis showed possible ethnic differences in effect. In East Asians (n=2,198 cases and 2,557 controls), the A allele was identified as the risk allele. Carriers of the AA genotype were 55% more likely to have essential hypertension compared to carriers of the AG and GG genotypes (summary OR=1.55, 95% CI: 1.14-2.09, p<0.01). Qualitative analysis suggested no association in Caucasians.</p> <p>Conclusions: Meta-analysis findings suggest that there is a likely ethnic difference in rs4149601 contribution to essential hypertension susceptibility. Areas for future research include further confirmation of ethnic differences and additional evaluation of the functional role of rs4149601 in blood pressure regulation and its clinical impact.</p>

Condensed abstract:

A loss-of-function variation (rs4149601) in *NEDD4L* has been implicated as a risk factor for essential hypertension, but inconsistently replicated. Meta-analysis found a null overall association between rs4149601 and essential hypertension (n=7 studies). However, subgroup meta-analysis restricted to East Asians (n=2,198 cases and n=2,557 controls) found a statistically significant association. Carriers of the AA genotype were 55% more likely to have essential hypertension compared to carriers of the AG and GG genotypes (summary OR=1.55, 95% CI: 1.14-2.09, $p<0.01$). Qualitative analysis found no association in Caucasians. Findings suggest that there is a likely ethnic difference in rs4149601 contribution to essential hypertension susceptibility.

ABBREVIATIONS DEFINITION LIST

All abbreviations are defined in-text.

TITLE PAGE

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Title:

Association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension: a systematic review and meta-analysis

Short Title: *NEDD4L* rs4149601 & hypertension

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The authors declare that they have no conflicts of interest.

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ABSTRACT AND KEYWORDS

Abstract:

Objective: A loss-of-function variation (rs4149601) in the ubiquitin protein ligase NEDD4-Like (*NEDD4L*) gene has been implicated as a risk factor for essential hypertension. However, this finding has been inconsistently replicated. A systematic review and meta-analysis was conducted to assess and synthesize available evidence on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism (SNP) and essential hypertension.

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Conclusions: Meta-analysis findings suggest that there is a likely ethnic difference in rs4149601 contribution to essential hypertension susceptibility. Areas for future research include further

confirmation of ethnic differences and additional evaluation of the functional role of rs4149601 in blood pressure regulation and its clinical impact.

Keywords:

Essential hypertension; NEDD4L; rs4149601; genetic association study; meta-analysis

TEXT

Introduction

Essential hypertension is a common and etiologically complex chronic disease with a strong heritable component [1]. A number of genes involved in blood pressure regulation have been postulated to be susceptibility candidates, including the ubiquitin protein ligase NEDD4-Like (NEDD4L) gene [2], a member of the HEC class of the E3 ubiquitin ligases. Through the epithelial sodium channel (ENaC)-NEDD4L-proteasome system, NEDD4L plays an important role in regulation of sodium transport in the kidneys [3] and the brain [4]. Therefore, it has been hypothesized that carriers of loss-of-function mutations in the *NEDD4L* gene, resulting in imbalance in sodium homeostasis, have increased risk of hypertension.

A cryptic splice site variant at the last nucleotide of exon 1 in the *NEDD4L* gene, the rs4149601 G/A single nucleotide polymorphism (SNP), has been implicated in the etiology of hypertension. Molecular studies showed that the A allele of rs4149601 fails to splice properly, resulting in a stop codon in exon 2, while the G allele generates an isoform with an evolutionarily novel C2 domain (isoform I). In addition, two common isoforms, one with an evolutionarily conserved C2 domain (isoform II) and one without a C2 domain (truncated isoform) are also generated [5]. The exact mechanism by which these isoforms act and how they interact to regulate blood pressure is unclear [6]. The first association studies of rs4149601 with hypertension were conflicting in identifying the risk allele, a phenomenon known as a “flip-flop” association [7]. In 2005, Russo et al. found that the A allele conferred significant risk for essential hypertension in African-Americans [8], but subsequent studies in Japanese males [9] and Swedes [10] (examining cross-

sectional and longitudinal blood pressure) found the G allele to be the risk allele. In East Asian populations, specifically in Chinese, the association of the rs4149601 SNP with essential hypertension was also inconsistently replicated [11-16].

The lack of consistent replication between studies may be due to the inadequate sample sizes of individual candidate gene association studies to detect weak associations (odds ratio [OR] of approximately 1.2), typically observed for common genetic variants such as rs4149601 [17]. Meta-analysis can be performed by combining these individual studies with insufficient statistical power (<80%) to obtain a more precise estimate of the true effect size [18].

A comprehensive appraisal and synthesis of the literature on the *NEDD4L* rs4149601 SNP and susceptibility to essential hypertension has not yet been reported. Therefore, this present study evaluated the association between rs4149601 and essential hypertension and identified the rs4149601 risk allele by systematic review and meta-analysis. Methodological issues of studies included in the meta-analysis and future recommendations for research involving rs4149601 and essential hypertension are discussed.

Methods

This systematic review and meta-analysis conformed to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (see Table, Supplemental Digital Content 1, containing the completed checklist) [19]. An unpublished review protocol was prepared for internal comment.

Search Strategy

PubMed (MEDLINE), EMBASE, and

<http://blogs.biomedcentral.com/bmcblog/2015/01/05/planning-a-systematic-review-think-protocols/>China National Knowledge Infrastructure (CNKI) literature databases were

searched from inception using the following keyword search string with corresponding

controlled vocabulary (i.e., MeSH, Emtree) additionally used when applicable: *hypertension*

and (*NEDD4L* or *NEDD4-2* or *NEDD4.2* or *RSP5* or *KIAA0439* or *rs4149601*) and

(*polymorphism* or *SNP* or *variation* or *mutation*). The HuGE Literature Finder database was

consulted for its listing of articles under the hypertension phenotype and *NEDD4L*. Reference

lists of articles included for review were hand searched for further relevant publications. No

publication date or language restrictions were imposed. The last electronic search was performed

on January 13, 2016.

Identification of Eligible Studies

Search results were screened independently by two authors (L.Z. and S.C.). Disagreements in

record screening classification between reviewers were resolved by discussion and until

consensus was reached. Level 1 screening consisted of evaluating information incorporated in

title, keywords, and abstract, returned by the electronic search. Level 2 screening consisted of

evaluating full-text reports for studies deemed potentially eligible after level 1 screening, or for

which insufficient information was available to determine eligibility (e.g., no abstract). For

multiple publications based on related data sets, the study with the greatest number of subjects

was included.

Studies were included for review if they met the following criteria:

- 1) Case-control, cohort or cross-sectional design
- 2) Clinically diagnosed essential hypertension
- 3) Investigated and reported on the association between rs4149601 and essential hypertension
- 4) Not molecular or animal research
- 5) Not review article, commentary/editorial, or conference abstract

Data Abstraction

The following information was extracted from studies included for review:

- 1) Author
- 2) Publication year
- 3) Country where the study was conducted
- 4) Subject characteristics (e.g., number of cases and controls, age, sex, and ethnic composition)
- 5) Case and control selection criteria
- 6) Genotyping method
- 7) Allele/genotype frequencies

Statistical Analysis

Meta-analysis was performed using OpenMeta[Analyst] (Tufts Medical Center, Boston, MA).

An *a priori* genetic model for the polymorphism was not assumed in the meta-analysis; the most appropriate model was indicated by comparison of summary ORs and 95% confidence intervals

(CIs), with the level of statistical significance (α) defined as a Z-test p -value of less than 0.05. Summary ORs for the allelic comparison (A vs. G) and genotypic comparisons of co-dominant (AG vs. GG and AA vs. GG), dominant (AA+AG vs. GG), and recessive (AA vs. AG+GG) genetics models were estimated using the inverse variance analysis approach, where the A allele was assumed to be the risk allele [8]. Between-study heterogeneity was quantified using the I^2 statistic [20] with a value $\geq 50\%$ indicating a high degree of between-study heterogeneity [21]. If low heterogeneity was observed ($I^2 < 50\%$), a fixed-effect model was used for the inverse variance method; otherwise, a random-effects (DerSimonian and Laird) model was used. Hardy-Weinberg equilibrium (HWE) was assessed in study controls using the χ^2 goodness-of-fit test.

Subgroup analyses by ethnicity and sex (due to a potential sex discrepancy in genetic risk at rs4149601 [9]) were planned to evaluate their effects on the association and as potential sources of between-study heterogeneity. A leave-one-out sensitivity analysis was conducted to check the robustness of summary ORs. Funnel plots were examined for evidence of publication bias. Furthermore, cumulative meta-analysis was conducted to detect temporal trends in effect [22].

Results

Study Inclusion and Characteristics

Study identification and screening process is summarized in Figure 1. Seventy-two studies were identified after deduplication and screened for eligibility. Eight studies were included for qualitative analysis [8,9,11-14,16,23] and seven studies (n=18,315 genotyped hypertensive cases and n=12,708 genotyped normotensive subjects) were included for meta-analysis [9,11-14,16,23]. One excluded case-control study conducted in Han Chinese examined the association

of interest, but did not report on the association due to significant deviation from HWE in controls ($p=0.0087$) [15], an indicator of potential selection bias, population stratification, or genotyping errors [24]. A case-control study conducted in the United States (US) was included in the qualitative analysis, but excluded from meta-analysis due to unreported allele/genotype frequency distributions; a significant association was reported in African-Americans and a non-significant association was reported in Caucasians (corresponding author did not respond to data request) [8]. A family-based study reporting a non-significant association between rs4149601 and essential hypertension in Greek Caucasians was relevant to the review, but excluded from analysis due to ineligible study design [25]. This study was excluded during level 1 screening, but referred to in the qualitative analysis.

Study characteristics are summarized in Table 1 and allele/genotype frequencies in Table 2. A majority of studies included for systematic review and meta-analysis were conducted in China ($n=5$) with two studies examining the association in Han Chinese [12,13] and one study each in Uyghur [11], Kazakh [14], and Mongolian [16] Chinese. The other studies were conducted in Japanese [9], Swedish Caucasians [23], US Caucasians and African-Americans [8]. No deviation from HWE was detected in the control subjects of studies included for meta-analysis. Only two articles differentiated genotype frequency distributions by sex [9,14], but one did not report the genotype frequency distribution for females due to lack of significant findings [9]. All studies were of case-control design except one cohort study [23]. The cohort study by Dahlberg et al. [23] only reported relative genotype distribution frequencies; therefore, genotype frequency distributions in cases and controls calculated from reported relative genotype frequencies for the overall study sample, adjusted for the reported genotyping call rate, assuming proportional SNP

genotyping failure rate across case-status and genotypes (see Table 2, footnote d). Note that (1) the misreported genotype frequencies in the Peng et al. study [16] were recalculated assuming the homozygous genotypes (AA and GG) were correctly reported (see Table 2, footnote c) and (2) Ishigami et al. study [9] did not report genotype distribution frequencies in female subjects. The corresponding authors for these studies did not respond to data confirmation inquiry.

Association between rs4149601 and Essential Hypertension

A non-significant association was found between the rs4149601 SNP and essential hypertension for the allelic and genotypic comparisons for all studies (n=7 studies; Table 3A). High between-study heterogeneity was detected in all comparisons ($I^2 > 50\%$). Sensitivity analysis confirmed the stability of the null summary effect estimates, with the large cohort study by Dahlberg et al. [23] exerting high influence (data not shown). Insufficient studies (n=1 study) reported sex-stratified genotype distributions to conduct subgroup analyses by sex.

Association between rs4149601 and Essential Hypertension by Ethnicity

In subgroup analysis restricted to studies conducted in East Asian populations (n=6 studies; n=2,198 genotyped hypertensive cases and n=2,557 genotyped normotensive subjects), meta-analysis found a statistically significant association between the rs4149601 SNP and essential hypertension for the recessive genetic model (Table 3B). Subjects with the homozygous AA genotype were 55% more likely to have essential hypertension compared to subjects with the AG and GG genotypes (summary OR=1.55, 95% CI: 1.14-2.09, $p < 0.01$) (Figure 2A). Low between-study heterogeneity was detected for this comparison ($I^2 = 26.0\%$) and the fixed-effect model was used for meta-analysis. The significant meta-analysis association is relatively robust; leave-one-

out sensitivity analysis found moderate influence on the combined effect by two studies [13,16] (Figure 3). Cumulative meta-analysis showed an initial null association trending to significance with accumulating evidence (Figure 2B). No overt publication bias was evident in the funnel plots (Figure 4).

Insufficient studies were available to conduct quantitative subgroup analysis for other ethnicities. However, all three reports in Caucasians [8,23,25], including a family-based study excluded from the present meta-analysis due to ineligible study design [25], are negative. One study of African-Americans reported a significant association [8].

Discussion

The present meta-analysis found a non-significant association between the *NEDD4L* rs4149601 SNP and essential hypertension in the overall analysis. There was also moderate-to-high between-study heterogeneity in the overall meta-analysis, reflective of the inconsistently reported findings between this SNP and hypertension (the so-called “flip-flop” phenomenon). However, when restricted to studies in East Asian populations, the summary ORs suggest a recessive genetic model effect for rs4149601, with the A allele as the putative risk allele. East Asian subjects with the homozygous AA genotype have a significantly increased likelihood of developing essential hypertension compared to subjects with the AG or GG genotypes. Individual study effects were generally consistent in direction and magnitude across several independent East Asian samples, with the ORs ranging from 1.49 to 1.95 in four of six included studies, with the A allele as the risk allele (Figure 2) [12-14,16]. Meta-analysis was not conducted for studies of Caucasian subjects, but three studies of different designs found no

association [8,23,25]. Considering the relative diversity of rs4149601 in different populations [26] and differences in hypertension risk in different ethnicities [27], the subgroup analysis findings suggest that the effect on hypertension risk conferred by rs4149601 may be inconsistent across ethnicities. Ethnic differences may partially explain the observed between-study heterogeneity and the “flop-flop” nature of the association between rs4149601 and essential hypertension.

There is biological plausibility for the increased risk of hypertension in individuals homozygous for the A allele. Molecular investigation found that while both alleles lead to the formation of the two common isoforms, one with an evolutionarily conserved C2 domain (isoform II) and one without a C2 domain (truncated isoform), the G allele also generates an isoform product with an evolutionarily novel C2 domain (isoform I), whereas the A allele only generates isoform II and the truncated product [5]. GG carriers would present with three isoforms, two with a C2 domain, and one without a C2 domain while AA carriers would present with only two isoforms, one with and one without a C2 domain [10]. The C2 is a calcium dependent domain involved in targeting NEDD4L to the plasma membrane where NEDD4L can ubiquitinate ENaC for subsequent proteasomal degradation. The rs4149601 is likely to impact on ENaC regulation, and therefore on sodium transport. Thus, it is plausible that homozygotes for the A allele, unable to produce the NEDD4L isoform I, are more susceptible to imbalances in blood pressure regulation leading to the development of hypertension. Preliminary findings of 546 Han Chinese patients with chronic kidney disease (not included in meta-analysis; conference abstract) also identified the AA genotype to be significantly associated with hypertension for the recessive model ($p=0.02$) [28].

Reporting quality among the included studies was generally inadequate compared to genetic association study reporting guidelines [29], particularly among the studies reported in the Chinese language [11,14,16]. Taking this into consideration, there were no serious limitations in the methodological design and implementation of the included studies. Although the diagnostic criteria for essential hypertension were defined relatively homogeneously across studies (Table 1), phenotype misclassification from white coat hypertension [30] or masked hypertension [31] may still be possible. None of the studies reported using 24-hour ambulatory blood pressure monitoring, the current state-of-the-art measurement of blood pressure. Misclassification errors in genotyping may be another source of bias, as SNP call rates were reported in two studies [13,23], implementation of genotyping quality control was reported in only one study [13], and phenotype blinding for genotyping was not reported in any of the included studies. Population stratification was not assessed in these studies.

A potentially negative direct association with essential hypertension in Caucasians notwithstanding, there is evidence that the rs4149601 SNP significantly influenced individual daytime blood pressure response to salt intake in Caucasian hypertensives [32], decreased blood pressure in Caucasians treated with β -blockers [33] or diuretics [33,34], and increased risk for adverse cardiovascular outcomes in Caucasians not treated with β -blockers [33] or diuretics [33,34]. These significant clinical findings showed that there are potential applications for rs4149601 in personalized medicine, including salt sensitivity screening, dietary modification and tailored drug therapy. Further confirmation and exploration of the aforementioned and other clinical outcomes are warranted, including considerations for ethnic differences. There is

preliminary evidence for an rs4149601 association with plasma aldosterone concentration in the additive model in Han Chinese [35]. Association with physiologically intermediate phenotypes, gene-gene and gene-environment interaction effects, especially salt intake, and mechanisms of *NEDD4L* isoforms action and interaction are areas requiring additional investigation.

The present meta-analysis re-evaluated genetic association studies of rs4149601 and essential hypertension identified by systematic review with enhanced statistical power. Despite a systematic study identification approach, the completeness of evidence may still be impeded by publication bias through the file drawer effect [36,37] and language bias [38]. The literature search conducted in the Chinese language database in addition to English language databases with no language restrictions minimized language bias. Deficiencies in reporting impeded meta-analysis efforts for the Caucasian subgroup [8,23], but individual study findings were consistently negative and allowed for qualitative synthesis across different study designs. Meta-analysis findings, in particular the significant subgroup finding for East Asians, can only be interpreted suggestively, due to the paucity of published evidence.

The synthesized findings suggesting a difference in effect of the *NEDD4L* rs4149601 SNP with hypertension across two broad ethnic categorizations is a stepping stone for new primary research. Future directions for research include confirmation of the differential relationship of rs4149601 and essential hypertension across ethnicities, the functional role of rs4149601 and its protein products in blood pressure regulation and hypertension pathogenesis, and the impact of this SNP on clinical outcomes, such as blood pressure responses to salt intake and antihypertensive medication use.

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FIGURE CAPTIONS

Figure 1. Flow diagram of the study identification and selection process.

Figure 2. Forest plot and cumulative meta-analysis plot on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension for the recessive genetic model (AA vs. AG+GG) in East Asians. (A) Meta-analysis was performed using an inverse variance random-effects (DerSimonian and Laird) model. The odds ratio for each study is represented in the plot as a black square with the area of the square corresponding to study weight. The 95% confidence interval is represented as a horizontal line. The summary odds ratio is symbolized as a diamond in the forest plot. (B) For the cumulative meta-analysis, a series of meta-analyses were performed where each meta-analysis in sequence incorporates one additional study to examine temporal trend in evidence.

Figure 3. Leave-one-out sensitivity analysis plot on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension for the recessive genetic model (AA vs. AG+GG) in East Asians.

Figure 4. A scatter plot of the standard error of the logarithm of the OR ($SE(\log[OR])$) against the OR of each study on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension for the recessive genetic model (AA vs. AG+GG) in East Asians. The relatively symmetrical inverted funnel shape is an indication of a lack of overt publication bias. Note that the effect included for the outlying study (indicated by *) by Ishigami

et al. [9] was restricted to male subjects; the overall study effect was reported as null (dashed arrow showing possible location for the overall study effect).

LIST OF SUPPLEMENTAL DIGITAL CONTENT

Supplemental Digital Content 1.pdf

Table 1. Characteristics of studies included in the systematic review and meta-analysis on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension

Study	Design	Genotyping method (validation method)	Ethnicity (Country)	Subject exclusion criteria	Hypertensive status	Diagnostic criteria	n	Female, %	Age, y	BMI, kg/m ²	SBP, mmHg	DBP, mmHg
Russo et al. (2005)	Case-Control	ABI Assays-on-Demand, Assays-by-Design, or SNPLEX	Caucasian & African-American (USA)	None.	Hypertensive	SBP>140 mmHg or antihypertensive medication use.	NR ^a	NR	NR	NR	NR	NR
					Normotensive	BP<140/90 mmHg, age ≥55 years, and no antihypertensive medication use.	NR ^a	NR	NR	NR	NR	NR
Ishigami et al. (2007)	Case-control	ABI BigDye Terminator Cycle Sequencing	Japanese (Japan)	Renal diseases, secondary hypertension, or diabetes mellitus.	Hypertensive	BP≥140/90 mmHg or antihypertensive medication use for >2 years and a family history of hypertension.	186	43.5	NR	NR	NR	NR
					Normotensive	BP<120/80 mmHg and no family history of hypertension.	181	34.3	NR	NR	NR	NR
Lian et al. (2008)	Case-control	ABI TaqMan PCR	Uyghur Chinese (China)	Evidence of intermarriage, alcohol abuse, or contraceptive use.	Hypertensive	BP≥140/90 mmHg or antihypertensive medication use.	344	42.4	54.0 (10.2)	28.1 (4.6)	163.5 (22.1)	95.8 (13.6)
					Normotensive	BP<140/90 mmHg and no history of hypertension	322	42.2	51.1 (10.2)	26.0 (4.2)	117.1 (11.5)	72.9 (7.8)

												and antihypertensive medication use.
Wen et al. (2008)	Case-control	ABI TaqMan PCR	Han Chinese (China)	Age <30 years, coronary heart disease, renal diseases, secondary hypertension, alcohol abuse, or contraceptive use.	Hypertensive	BP \geq 140/90 mmHg or antihypertensive medication use.	272	59.6	56.2 (11.3)	25.9 (3.7)	157.3 (19.7)	99.0 (15.9)
					Normotensive	BP<135/85 mmHg and not previously diagnosed as hypertensive.	356	62.1	49.5 (12.8)	24.1 (3.2)	117.3 (12.0)	75.9 (7.4)
Luo et al. (2009)	Case-control	PCR-RFLP (10% samples randomly selected for bidirectional sequencing)	Han Chinese (China)	Evidence of previous stroke, coronary heart disease, diabetes mellitus, renal diseases, or secondary hypertension.	Hypertensive	BP \geq 140/90 mmHg on 3 occasions within 2 months or taking antihypertensive medication.	833	68.3	58.3 (8.4)	26.0 (3.5)	168.0 (22.6)	99.3 (12.1)
					Normotensive	BP<140/90 mmHg and no family history of hypertension.	853	68.2	56.3 (7.9)	24.4 (3.5)	122.6 (11.2)	79.3 (6.8)
Wang et al. (2010)	Case-control	ABI TaqMan PCR	Kazakh Chinese (China)	Evidence of secondary hypertension.	Hypertensive	BP \geq 140/90 mmHg on 3 occasions or antihypertensive medication use.	383	55.4	46.2 (7.6)	28.1 (4.5)	150.6 (25.9)	96.9 (10.9)
					Normotensive	BP<140/90 mmHg and no history of antihypertensive medication use.	500	59.2	42.0 (7.4)	25.3 (3.6)	117.8 (11.1)	78.3 (7.5)
Dahlberg et al. (2014)	Cohort	ABI TaqMan PCR	Caucasian (Sweden)	None.	Hypertensive	BP \geq 140/90 mmHg or antihypertensive	16912 ^b	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c

					medication use.							
					Normotensive	Non-hypertensive subjects	10652 ^b	NR ^c	NR ^c	NR ^c	NR ^c	NR ^c
Peng et al. (2014)	Case-control	ABI TaqMan PCR	Mongolian Chinese (China)	Evidence of intermarriage, alcohol abuse, or contraceptive use.	Hypertensive	BP \geq 140/90 mmHg or antihypertensive medication use.	308	NR ^d	NR	NR	NR	NR
					Normotensive	BP<140/90 mmHg and no history of hypertension and antihypertensive medication use.	454	NR ^d	NR	NR	NR	NR

ABI, Applied Biosystems Inc.; BP, blood pressure; DBP, diastolic blood pressure; n, recruited sample size; NR, not reported; PCR, polymerase chain reaction; PCR-RFLP, PCR-restriction fragment length polymorphism; SBP, systolic blood pressure. Values are presented as mean (SD), unless otherwise specified.

^a Not reported in the article on the association of interest, but the sample was previously reported to include 193 Caucasian subjects (100 cases and 93 controls) and 218 African-American subjects (120 cases and 98 controls) [39].

^b Calculated using reported relative frequency distribution by hypertension status from total study sample (n=27,562).

^c Subject characteristics reported only for the overall study sample: Female, %: 60.2; Age, y: 58.0 (7.6); BMI, kg/m²: 25.8 (4.0); SBP, mmHg: 141.2 (20.1); DBP, mmHg: 85.6 (10.0).

^d Authors noted in their report that both sexes were recruited and analyzed, with controls randomly recruited from the population.

Table 2. Genotype distributions of studies included in the meta-analysis on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension

Study	Ethnicity (Country)	Subgroup	Cases					Controls					
			n	RAF (A)	Genotypes			n	RAF (A)	Genotypes			HWE <i>p</i>
					AA	AG	GG			AA	AG	GG	
Ishigami et al. (2007) ^a	Japanese (Japan)	East Asian	105	0.124	2	22	81	119	0.210	6	38	75	0.6795
Lian et al. (2008) ^b	Uyghur Chinese (China)	East Asian	311	0.164	6	90	215	303	0.183	10	91	202	0.9492
Wen et al. (2008) ^b	Han Chinese (China)	East Asian	266	0.158	7	70	189	350	0.170	5	109	236	0.0527
Luo et al. (2009)	Han Chinese (China)	East Asian	833	0.232	38	311	484	853	0.188	22	276	555	0.0718
Wang et al. (2010) ^b	Kazakh Chinese (China)	East Asian	375	0.173	15	100	260	478	0.174	13	140	325	0.6526
Dahlberg et al. (2014) ^d	Caucasian (Sweden)	Caucasian	16,117	0.353	1,957	7,456	6,703	10,151	0.353	1,273	4,628	4,250	0.8118
Peng et al. (2014) ^c	Mongolian Chinese (China)	East Asian	308	0.282	35	104	169	454	0.250	28	171	255	0.9252
Total (Overall)			18,315		2,060	8,153	8,101	12,708		1,357	5,453	5,898	
Total (East Asian)			2,198		103	697	1,398	2,557		84	825	1,648	

HWE, Hardy-Weinberg equilibrium; RAF, risk allele frequency; n, genotyped sample size; NR, not reported.

^a Genotype frequency distribution only reported for male subjects. Overall subjects: 186 hypertensive cases and 181 normotensive controls. Authors reported null associations for both overall and female subjects.

^b Genotyped sample size is less than the recruited sample size due to a SNP call rate of <100%.

^c Genotype frequency distributions misreported in original publication; recalculated assuming homozygous genotype frequencies were correctly reported.

^d Genotype frequency distributions in cases and controls calculated from reported relative genotype frequencies for the overall study sample (AA: 12.3%, AG: 46.0%, GG: 41.7%) and for cases (AA: 60.6%, AG: 61.7%, GG: 61.2%), adjusted for reported 95.3% genotyping call rate, assuming proportional SNP genotyping failure rate across case-status and genotypes (total study sample, n=27,564).

Table 3. Meta-analysis results on the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension

Comparison		Number of studies	OR (95% CI)	<i>p</i> -value	Effect model	<i>I</i> ² (%)
(A) Overall						
Allelic	A vs. G	7	1.01 (0.89-1.16)	0.84	Random	68.2
Co-dominant model	AG vs. GG	7	0.97 (0.85-1.12)	0.70	Random	52.7
	AA vs. GG	7	1.24 (0.85-1.83)	0.27	Random	64.6
Dominant model	AA+AG vs. GG	7	0.99 (0.86-1.15)	0.91	Random	60.5
Recessive model	AA vs. AG+GG	7	1.25 (0.86-1.83)	0.24	Random	64.0
(B) East Asian						
Allelic	A vs. G	6	0.99 (0.82-1.21)	0.96	Random	69.7
Co-dominant model	AG vs. GG	6	0.93 (0.75-1.14)	0.47	Random	60.3
	AA vs. GG	6	1.54 (1.14-2.09)	<0.01	Fixed	43.5
Dominant model	AA+AG vs. GG	6	0.95 (0.76-1.19)	0.68	Random	66.5
Recessive model	AA vs. AG+GG	6	1.55 (1.14-2.09)	<0.01	Fixed	35.9

CI, confidence interval; OR, odds ratio.

Figure 1. Flow diagram of the study identification and selection process.

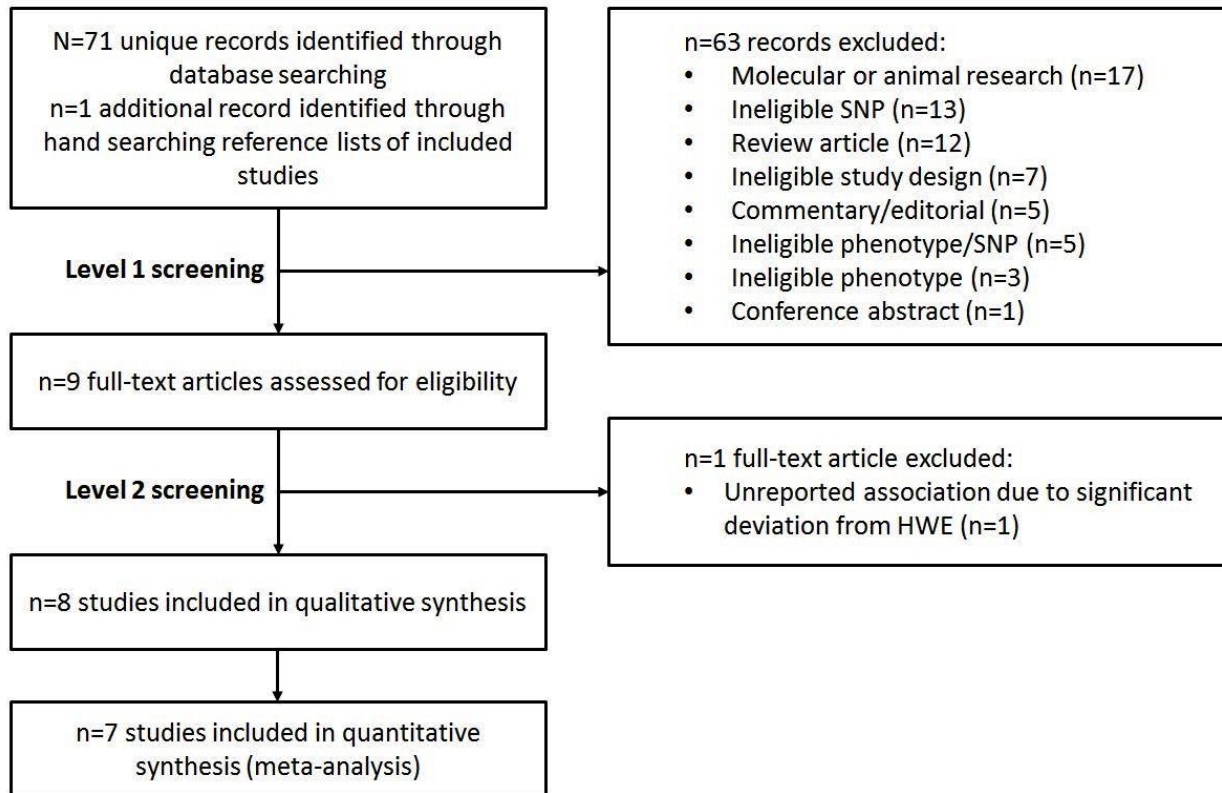


Figure 2. Forest plot of the association between the *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension for the recessive genetic model (AA vs. AG+GG).

Meta-analysis was performed using an inverse variance (IV) fixed-effect model. The odds ratio for each study is represented in the plot as a black square with the area of the square corresponding to study weight. The 95% confidence interval (CI) is represented as a horizontal line. The summary odds ratio is symbolized as a diamond in the forest plot.

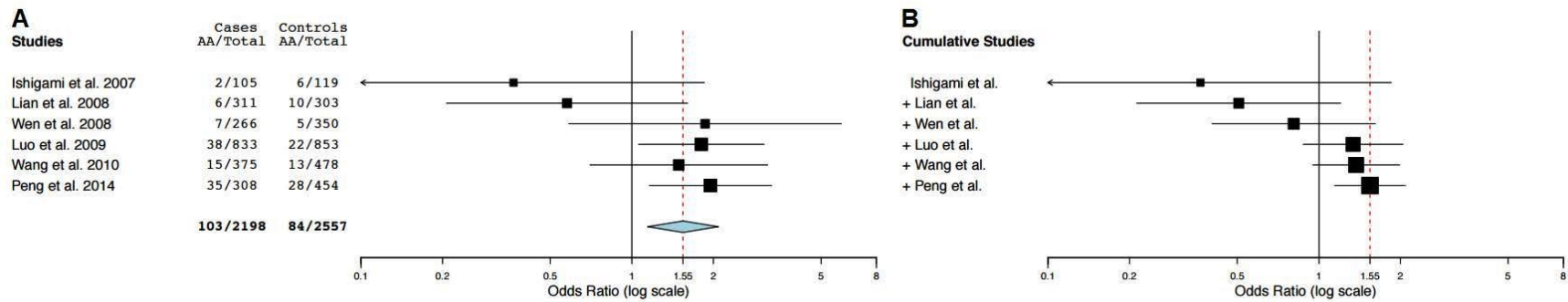


Figure 3.

Studies

Overall

- Ishigami et al.
- Lian et al.
- Wen et al.
- Luo et al.
- Wang et al.
- Peng et al.

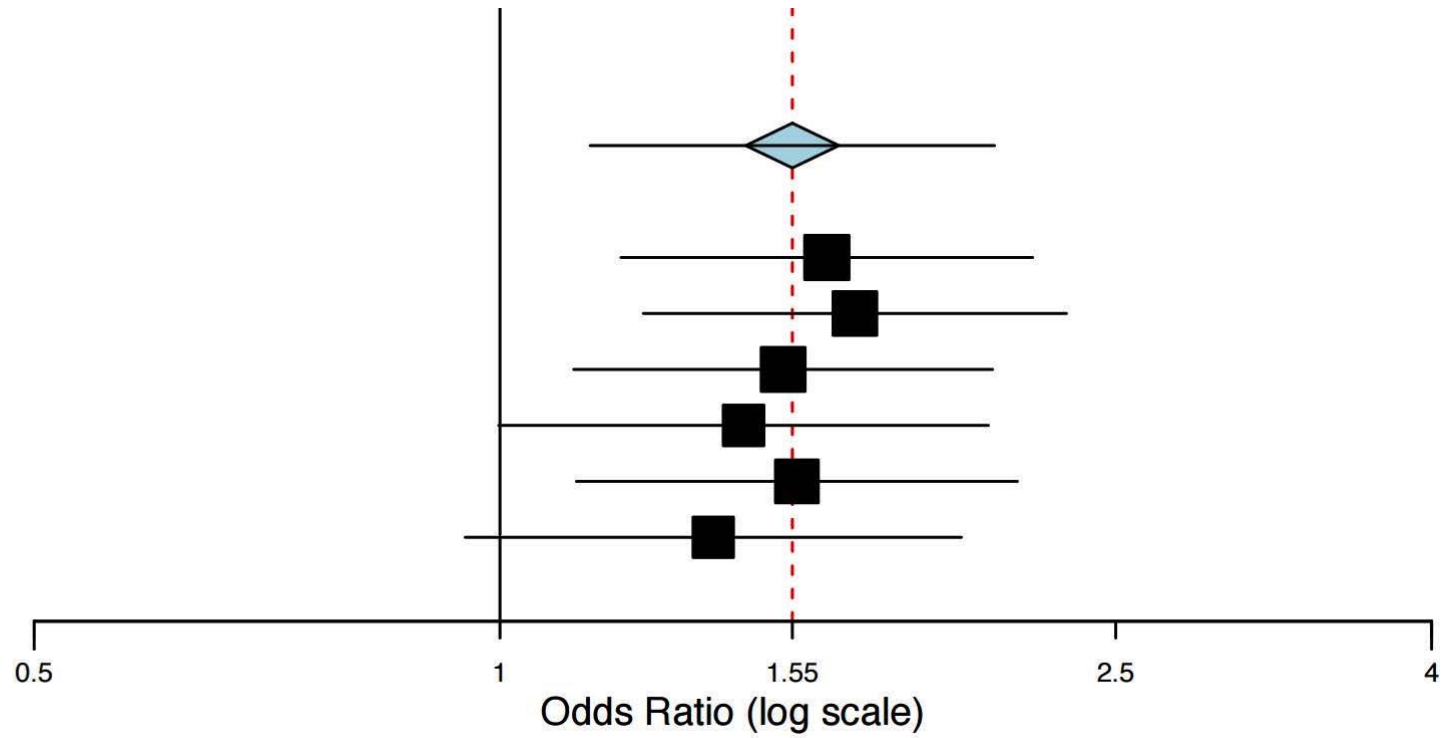
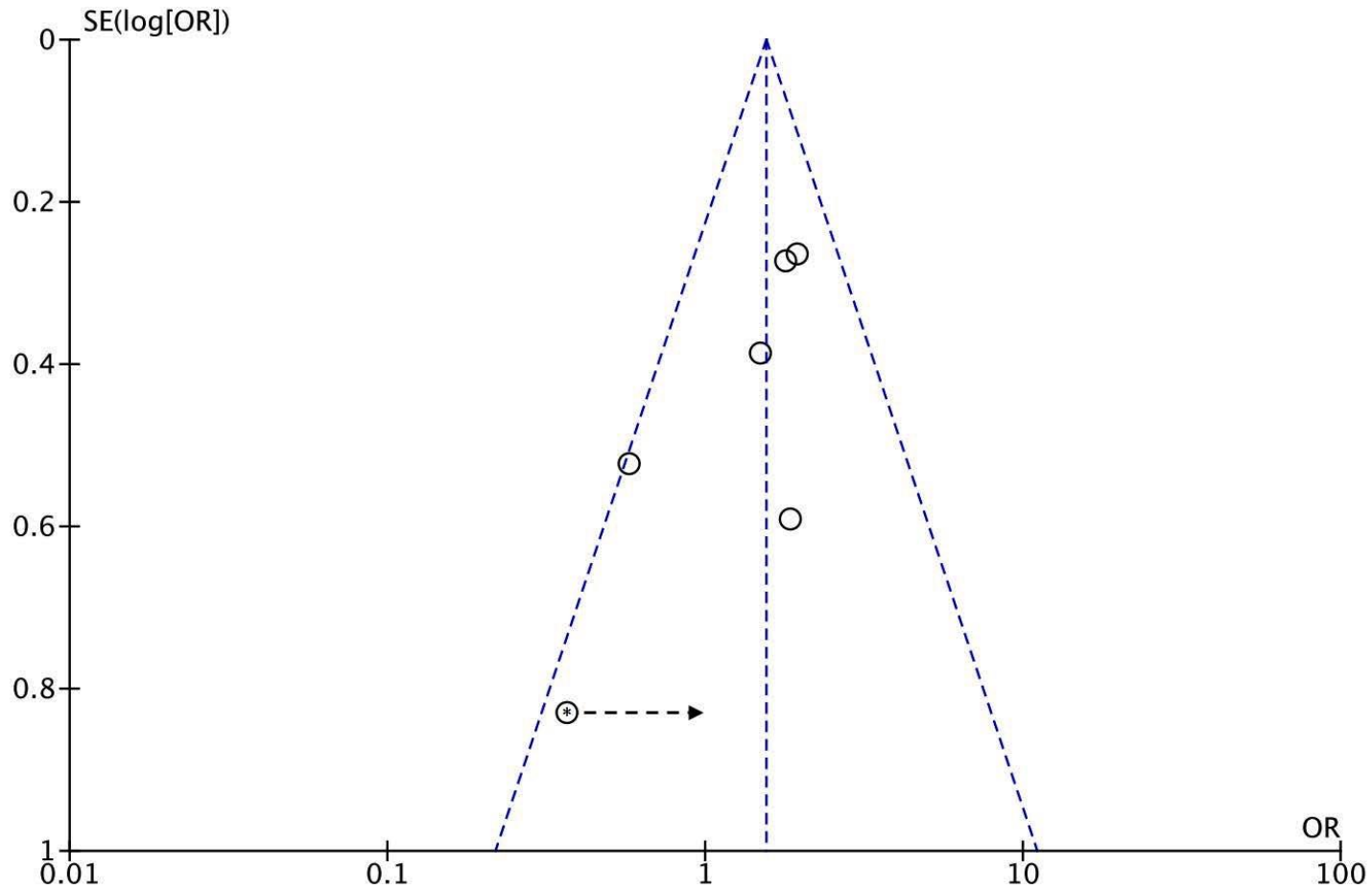


Figure 4. *NEDD4L* rs4149601 single nucleotide polymorphism and essential hypertension for the recessive genetic model (AA vs. AG+GG).

The graph is a scatter plot of the standard error of the logarithm of the OR ($SE(\log[OR])$) against the OR of each study. The relatively symmetrical inverted funnel shape is an indication of a lack of overt publication bias.



11.3.4 CSEB-SCEB Conference Abstract

11.3.4.1 Abstract #114 for oral presentation on June 10, 2016.

Conference: The Canadian Society for Epidemiology and Biostatistics National Student Conference 2016

Authors: Stephen Kutcher, Dr Frans H.H. Leenen M.D., Ph.D., Dr Alexander Stewart Ph.D., Hannah Nicolas, & Dr Frédérique Tesson Ph.D.

Title:

The influence of the rs4149601, rs2288774, and rs576416 *NEDD4L* single nucleotide polymorphisms in the development of salt sensitive hypertension with age in a Canadian Caucasian population.

Background:

Hypertension, a leading risk factor for cardiovascular disease, exhibited in 17.7% of the Canadian population, is influenced by the environment and genetics. Salt-sensitivity is described at higher rates in the hypertensive population. The NEDD4-like (*NEDD4L*) protein is important in sodium reabsorption and has been implicated in essential hypertension and salt-sensitivity.

Objectives:

Two variations (rs4149601/rs2288774) found in *NEDD4L* have been associated with salt sensitivity and hypertension; a third (rs576416) is in linkage disequilibrium with rs4149601. The purpose of this study is to assess the relationship between *NEDD4L* rs4149601, rs2288774, and rs576416 single nucleotide polymorphisms with sodium and age on blood pressure (BP).

Methods:

Hypertensive patients were recruited through the University of Ottawa Heart Institute. Eligible subjects were studied off anti-hypertensive medications. Daytime BP was measured using 24hr ambulatory BP monitoring in 190 Caucasian hypertensives (BP \geq 130/85 mmHg). 24hr urine Na⁺ was collected. DNA was genotyped on the GeneTitan Affymetrix Axiom platform and through TaqMan MGB probe-based RT-PCR. Simple and multivariate linear regression modelling with SAS 9.4.0 was used for genotypic comparisons affecting BP, combined with age and corrected urine sodium.

Results:

Multiple linear regressive modelling failed to identify an overall statistical model that significantly described a positive relationship when analyzing the discrete association of the GG rs4149601, CC rs2288774, and AA rs576416 genotypes and BP in a modest sample size (n=190) aged 22 to 61 years. However, in the male hypertensives (n=120) the combination of the GG rs4149601 and AA rs576416 ($\beta=0.021$, $P=0.03$) and the GG rs4149601 and CC rs2288774 ($\beta=0.020$, $P=0.04$)

genotypes showed significant associations with BP in borderline significant models (P=0.055 and P=0.094 respectively), when analyzed with urine sodium levels and age.

Conclusions:

Multiple linear modelling describing borderline significant findings in the interaction of rs4149601 with rs576416, and rs4149601 with rs2288774 in male hypertensives suggests of the possible synergism between polymorphisms and development of salt-sensitive hypertension. Future research could evaluate the role of NEDD4L on the sex differences in early-onset salt-sensitive hypertension.

Disclosure:

No conflicts of interest. Funding from the Canadian Institutes of Health Research (CIHR) and the Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST).

11.3.4.2 Reviewers comments for Abstract #114

Abstract Title: The influence of the rs4149601, rs2288774, and rs576416 NEDD4L single nucleotide polymorphisms in the development of salt sensitive hypertension with age in a Canadian Caucasian population.

Abstract # 114

Rating Categories:

Rating scores are averaged over all abstract reviews on a scale of 1=disagree strongly to 5=agree completely for each of the following criteria.

Research question is important and relevant: 3.67

Methods appear rigorous and appropriate: 4

Project provides new insights or knowledge: 3.67

Abstract is clear and well organized: 4

Overall rating of the abstract: 3.67

Additional Comments:

a study of modest size, with finding only in a subset, thus of uncertain clinical significance. Methodologically, a high quality-study with some interesting findings.

The End.

“To infinity... and beyond!” – *Buzz Lightyear*