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DISCERNING THE EFFECT OF SALT, RIBOFLAVIN AND MTHFR ON ACUTE BLOOD PRESSURE
RESPONSE

By

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ABSTRACT OF THE DISSERTATION

Discerning the Effect of Salt, Riboflavin and MTHFR on Acute Blood Pressure Response

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Dissertation Directors:

Joshua W. Miller and Paul A.S. Breslin

Problem: Elevated blood pressure (BP) is a top cause of mortality and leads to millions of global deaths each year; yet the cause of general hypertension (HTN) is not known. Many studies link dietary factors, particularly high sodium levels, with HTN and related cardiovascular diseases. Other studies have identified certain genes, including methylenetetrahydrofolate reductase (MTHFR) involved in folate metabolism, with a greater risk of developing HTN.

Hypothesis: These studies sought to identify the impact of MTHFR genotype on the acute BP response to salt ingestion. We hypothesized that individuals would have a greater decrease in BP following salt ingestion compared to water and that this response would be diminished by the MTHFR 677TT variant allele.

Methods: In an initial cohort, acute BP response was assessed, multiple times per subject, in a series of time points following an ingestion of salt or water. A secondary cohort was formed of individuals with MTHFR677 genotypes whose BP and blood were collected. Blood samples were analyzed for plasma riboflavin, homocysteine, MTHFR genotype and nitrate/nitrite levels. These data were analyzed for differences in BP response, genotypes, micronutrient status and for associations between subjects' characteristics and BP response.

Results: BP decreases within the first 60 minutes following salt ingestion. When measuring resting BP, no difference was found in BP between MTHFR genotypes. However, individuals with the 677TT variant genotype demonstrated an attenuated acute BP response to salt ingestion compared to individuals with the wild-type 677CC genotype.

Conclusions: BP decreases acutely in response to salt ingestion. The MTHFR 677TT variant genotype was not associated with elevated resting BP. However, measuring acute BP response illustrated a limited response in the MTHFR variant genotype compared with the wild-type MTHFR genotype. These findings suggest that acute BP response may be a useful measure of a dynamic system, highlighting characteristics that are risk factors for HTN.

ACKNOWLEDGEMENT AND DEDICATION

Dedication:

For my father, who told me: “Your best is all you can do.”

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To write the names of everyone who influenced or contributed to my completing this dissertation would fill another 100 pages. Know that you are appreciated and my debt to you is great.

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INTRODUCTION

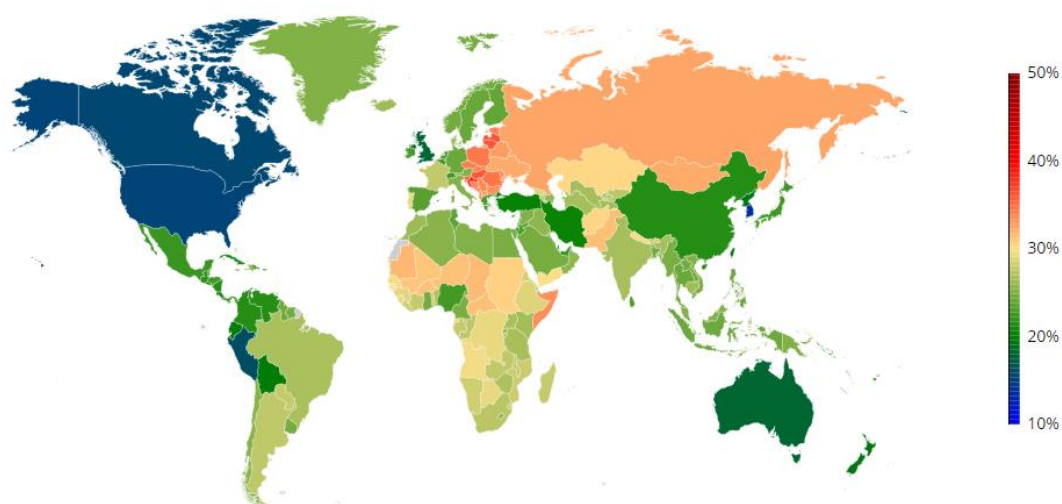
Global Impact of Blood Pressure

To accomplish the American Heart Association's 2020 goal of improving cardiovascular health of all Americans, medical professionals will need to consider risk factors broadly related to stroke and heart disease including elevated blood pressure (hypertension) [1]. In 2012, cardiovascular disease (CVD) was the leading cause of US death and essential hypertension was among the top ten causes [2, 3]. The global burden of death attributed to hypertension (HTN) is largest among all other diseases globally [4]. An Individual's blood pressure increases steadily with age [5] which is exacerbated by chronic high salt intake; and hypertension is a risk factor for cardiovascular diseases [6]. The impact of hypertension (HTN) goes beyond its link to CVD and is also associated with diabetes [7], metabolic syndrome [8], pre-eclampsia [9-11], dementia [12-14] and depression [15-17].

Dietary guidelines over many years have made recommendations specifically designed to reduce risk for HTN, and yet the incidence continues to increase [18]. Additionally, global dietary HTN prevention strategies are currently criticized for lack of beneficial effect [19, 20]. The crux of this problem is the growing number of affected individuals and the small amount of information about how to prevent increases in BP.

In westernized countries, the number of people with elevated BP and HTN is growing rapidly [21]. In developing countries, the prevalence is higher than the global rate [22-24]. While the rate dropped between 1998 and 2008 to less than 40% globally, the number of people is greater at the end of that 20 year span [25]. As countries begin introducing western diets, metabolic diseases increase and cause related issues also affecting BP [26]. Efforts to reduce this health burden have been unsuccessful [27], and new science-based strategies to treat HTN are needed [28].

Figure 1: 2015 Global Elevated BP in Men



Scale on right illustrates percentage of male population with elevated BP in each country. Blue is lowest and deep red is highest. Elevated BP means SBP ≥ 140 mmHg or DBP ≥ 90 mmHg [29]

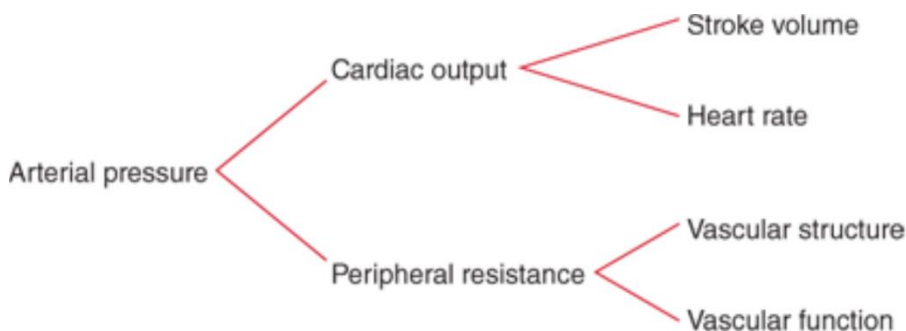
Blood Pressure Definition

BP is a measurement of the pressure against the walls of vessels in which blood circulates [30]. BP is measured as the pressure when the heart is contracting and pressure when the heart is at rest. The former is the systolic pressure (SBP) and the latter is the diastolic pressure (DBP) [31]. Often an average of these two is used called mean arterial pressure (MAP). MAP is equal to $DBP + 1/3 (SBP - DBP)$ [32]. This average provides a one number overview of the

pressure in the circulatory system [33]. Both SBP and DBP have been associated with risk for HTN and CVD and both are considered important measures in the study of these conditions [34, 35].

BP is determined by the health of the heart and of the vascular system. An indicator of heart health is cardiac output (CO) and the health of the vascular system is influenced by peripheral resistance and blood volume as shown in figure 2 [36-38]. These processes are monitored and controlled via the autonomic nervous system through a pathway of sympathetic and parasympathetic nerve fibers connected to the sinoatrial node in the heart [39-41].

Figure 2: Determinants of Arterial Pressure



Arterial pressure is determined by cardiac output and peripheral resistance [36].

CO is the amount of blood pumped out of the heart in a specific amount of time and is approximately 5.0L/min in a supine, healthy man [42]. CO varies due to changes in heart rate, which is how frequently the heart pumps out blood, and stroke volume, which is the amount of blood pumped out of the heart in a beat. Eating, exercise, excitement and high temperature all increase CO, while sitting/standing from a lying position, heart disease and irregular heart rate patterns decrease CO [42]. CO directly influences BP by changing the amount of blood that flows through the vessels [43].

Peripheral resistance is a measure of the resistance of vessels to the flow of blood [37]. In normal circulation, a thin layer of blood remains in contact with the vessels and speed of the blood increases toward the center of the vessel [44]. Blood with higher concentration of certain proteins or with red blood cells that are inflexible can create a turbulent flow which is slower than a healthy flow [45]. This causes a change in the pressure of the vessels and also requires more work of the heart to compensate [46, 47].

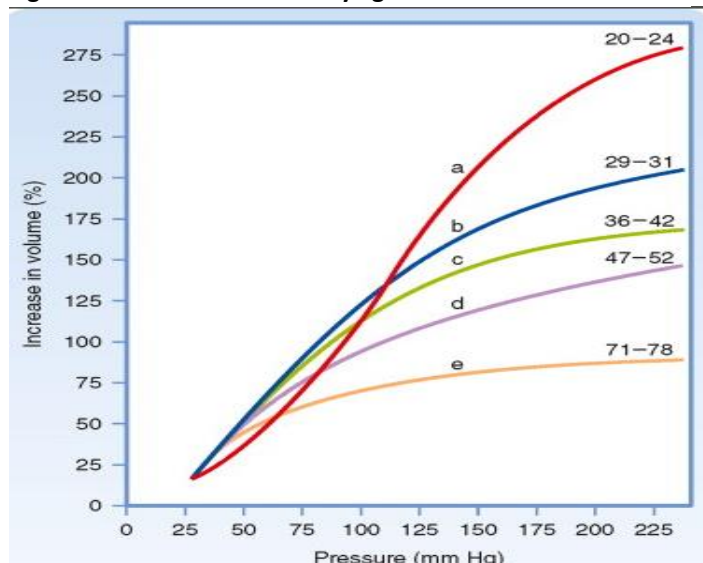
CO and peripheral resistance are both influenced by fitness of the heart and vessels. Stroke volume correlates with the strength of the heart's contraction and decreases as the cardiac muscle fiber strength weakens [48]. If the vessels become inflexible or do not respond to dilation and constriction, this increases peripheral resistance. Maintaining healthy heart muscles and blood vessels is critical to maintaining healthy BP [49, 50].

The pressure in the circulatory system is also affected by the efficiency of the pulmonary and renal systems [51-53]. The rate at which oxygen diffuses into the blood and carbon dioxide back to the lungs influences CO [54]. If parts of the body require more oxygen, the lungs will work to diffuse more air and the heart can increase its rate and stroke volume [54]. The kidney aids in maintaining balance of electrolytes in the blood by filtering or conserving sodium, potassium and calcium [55, 56]. The pressure in the vascular system triggers either constriction or dilation of renal vasculature and increases or decreases glomerular filtration rate [57]. Dysregulation in these pulmonary and renal processes can result in HTN [58-61]

Individuals in a population have a varying range of BP, impacted by age, sex, environment and genes. The strength of the muscles in the heart diminishes, reducing cardiac output [43]. As mentioned above, the flexibility of the arteries can also lessen, causing increases in pressure and/or peripheral resistance. As the health of the system diminishes, the ability to accommodate large changes in blood volume also diminishes as shown in Figure 3. As

individuals age, the amount of volume that can be accommodated at a healthy BP lessens, so changes beyond that can be damaging to the vascular system [62]. Large fluctuations in blood volume (and resulting CO) and peripheral resistance that cannot be regulated effectively may be one cause of HTN.

Figure 3: Volume to BP ratio by age



At younger ages, a larger range of blood volume can be accommodated by changing BP. At older ages, lesser increases in blood volume are related to much higher BP [44, 63, 64].

HTN is defined as chronic elevated BP. Healthy BP is between 90 and 120 mmHg SBP and between 60 and 80 mmHg DBP. As an individual's average SBP or DBP increases above these numbers, they are considered pre-hypertensive or hypertensive with increasing severity [65]. Table 1 illustrates the categorization of individuals from normal BP to severe hypertension [66]. Individuals with SBP below 90 mmHg or DBP below 60 mmHg are diagnosed as hypotensive [67]. HTN is associated with development of cardiovascular disease and other heart complications [68]. In addition, HTN is one of the key elements of metabolic disease and frequently develops in patients with diabetes [69, 70] and osteoporosis [71, 72]. Hypotension is also associated with diseased conditions including injuries from fainting falls, and insufficient

oxygen to brain and extremities [73, 74]. Maintaining BP in a healthy range (SBP 90 to 120 and DBP 60 to 80) is important to cardiovascular health.

Blood Pressure Category	Systolic mm Hg (upper #)		Diastolic mm Hg (lower #)
Hypotensive	Less than 90	and	Less than 60
Normal	90 - 120	and	60 - 80
Prehypertension	120 – 139	or	80 – 89
High Blood Pressure (Hypertension) Stage 1	140 – 159	or	90 – 99
High Blood Pressure (Hypertension) Stage 2	160 or higher	or	100 or higher
Hypertensive Crisis (Emergency care needed)	Higher than 180	or	Higher than 110

Table 1: Blood Pressure Category Definitions

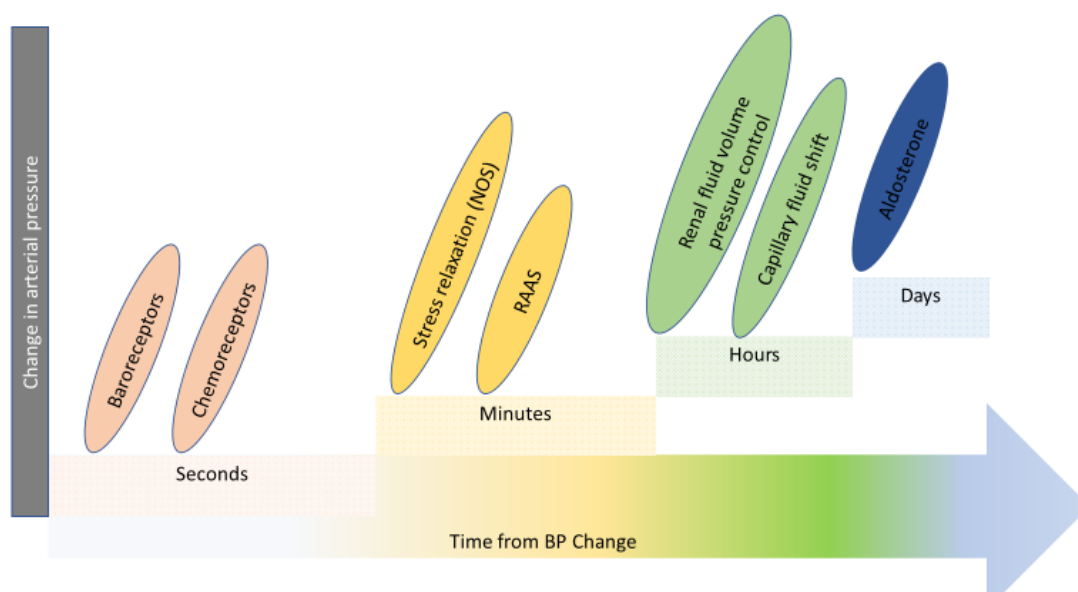
Healthy BP is 90 – 120 mmHg over 60-80 mmHg. Above or below this range is unhealthy [66].

Blood Pressure Regulation

BP is a measure of a system that changes with environmental influence. Diet, exercise and stress all influence BP. Changes can be sudden (i.e. quick postural change) or long term (i.e. changes in system physiology). Maintaining healthy BP is dependent on effective regulation of these changes, and the body has different systems in place to monitor BP levels and maintain them as close to baseline as possible.

The mechanisms that regulate BP act within seconds, minutes, hours or days of a pressure change as shown in Figure 4. Within seconds, baroreceptors respond directly to pressure in the vessels. Later actors include the Renin-Angiotensin-Aldosterone system (RAAS), nitric oxide (NO) production and Atrial Natriuretic Peptides [75]. Over a longer period, the balance between pressure and kidney regulation affects the resting systemic BP. Some of these responses are through the CNS and others act in response to hormone and volume sensors [64].

Figure 4: Regulators of BP by Time of Response



Various mechanisms regulate BP. These changes act within seconds or up to days after a pressure change.

Based on [76].

Within Seconds: Baroreceptors

Baroreceptors are the BP regulatory mechanism that responds immediately to BP change [77]. These receptors respond to high or low pressure in the blood vessels and send

direct signals to the brain [78]. These stretch

receptors in the heart and vessel walls respond

as pressure rises and causes distension. The

receptors signal this pressure change by rapidly

discharging, thus preventing increases in vagal tone

and initiating vasodilation and reduced cardiac

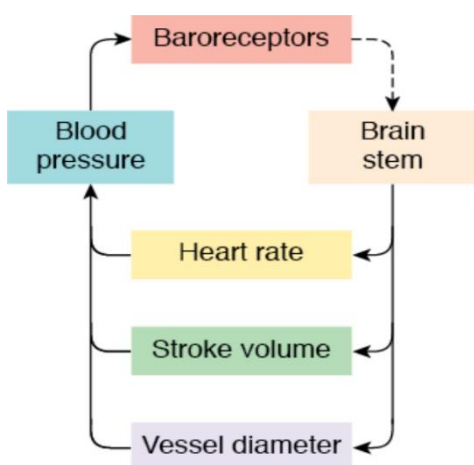
output [79], shown in Figure 5 [80]. Baroreceptors

reset quickly, and at an elevated pressure in HTN

[81], so are not effective as a long-term

regulator [82].

Figure 5: Baroreceptors and BP



Baroreceptors signal reflexes that affect cardiac output and vasodilation, resulting in BP regulation [80].

Within seconds: Chemoreceptors

Another rapid acting regulator are chemoreceptors which respond to reduction in partial pressure of oxygen and increase in partial pressure of carbon dioxide [79]. These receptors mainly cause changes in respiration, but also trigger vasoconstriction.

Within Minutes: NOS

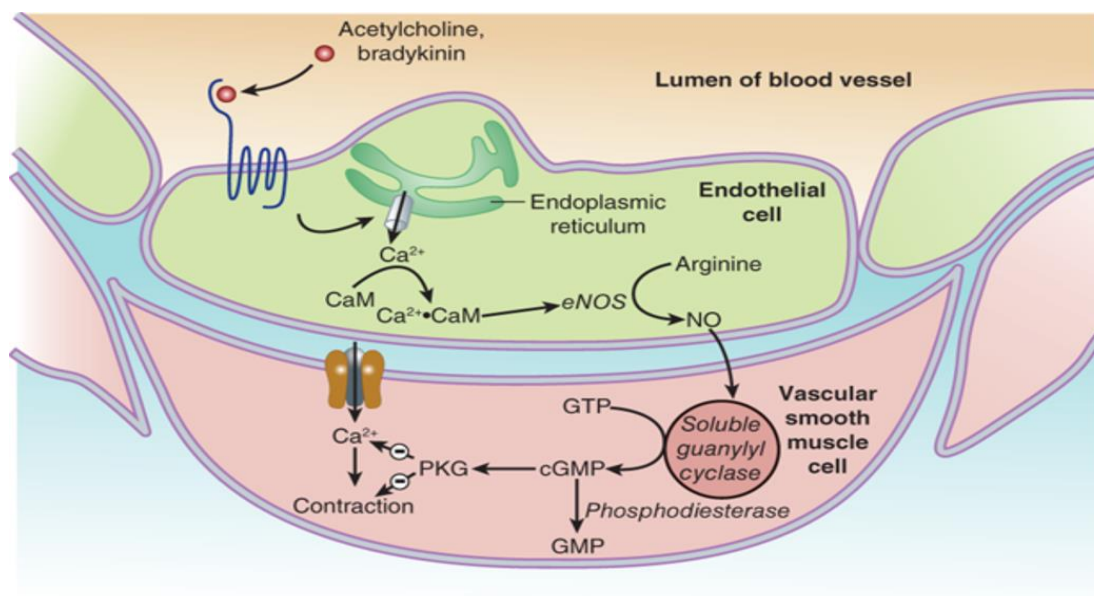
The NO production pathway is responsible for vasodilation of blood vessels within minutes of an increase in arterial pressure. NO Synthase (NOS) is the enzyme that produces NO (chemical structures in Appendix A1). This enzyme's three conserved forms (50% amino acid identity) are disbursed through key areas in the body: (1) inducible (iNOS) found throughout the body; (2) endothelial (eNOS) found in blood vessels and vascular tissue; and (3) neuronal (nNOS) found in neuronal tissue [83, 84]. Minor differences in these isoforms allow selective pharmacological intervention [85]. The three isoforms are similar in structure and function; however, iNOS is constitutively active while eNOS and nNOS are activated by binding with calcium and calmodulin [86-90].

NOS is a homodimeric enzyme, with each subunit containing a reductase and an oxygenase domain. Cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are bound in the reductase domain and cofactor tetrahydrobiopterin (BH_4) binds with the oxygenase domain [91, 92]. The two subunits are stabilized by BH_4 attaching at a hydrophobic core near the substrate, L-arginine, binding site [93, 94]. The enzyme's hinge moves through calcium/calmodulin binding which allows NO production [95].

Calcium is available in the endothelial cell through action of acetylcholine and bradykinin [96]. When calcium levels saturate the cell and calcium binds with calmodulin, activation of NOS causes an electron transfer across the cofactors [97]. Electrons flow from the reductase domain of one subunit to the oxygenase domain of the opposite subunit to form NO

and L-citrulline from L-arginine [98]. Once produced, NO exits the endothelial cell and enters smooth muscle cells [96]. NO activates a G-coupled protein cascade which inhibits calcium dependent muscle contraction [99]. This pathway is illustrated in Figure 6 [100].

Figure 6: Pathway of Nitric Oxide Production



NOS is activated by acetylcholine or bradykinin and produces NO which enters vascular smooth muscle cells and triggers production of cGMP [100].

There are multiple ways that NO can be intercepted or inactivated, once produced. For example, NO is highly reactive with metals and O_2 , so higher concentrations of these in the blood may reduce the impact of NO [86, 101]. NO reacts with other NO molecules or heme groups to form decomposition products, mostly nitrite (NO_2^-) and nitrate (NO_3^-) [99]. These downstream products do not carry the same vasodilatory effects that NO does, but in small quantity NO_2^- can be reduced to NO [102, 103]. However, NO can also react with reactive oxygen species to form peroxynitrite (ONOO^-) and damaging free radicals that cause oxidative stress [104]. These reactions with NO limit the vasodilatory effects of NO production.

NO has a short life of just 1 to 4 seconds in red blood cells and only a few milliseconds in whole blood [105, 106]. Therefore, it is challenging to measure NO levels directly. NO_2^- can last

approximately 10 seconds and NO_3^- can last up to minutes in whole blood [107, 108]. Plasma NO_2^- levels typically range from 1-13 $\mu\text{mol/L}$, while plasma NO_3^- range from 4-45 $\mu\text{mol/L}$ [109]. These two products are used frequently to assess NO levels [110, 111] to study its effect on BP regulation.

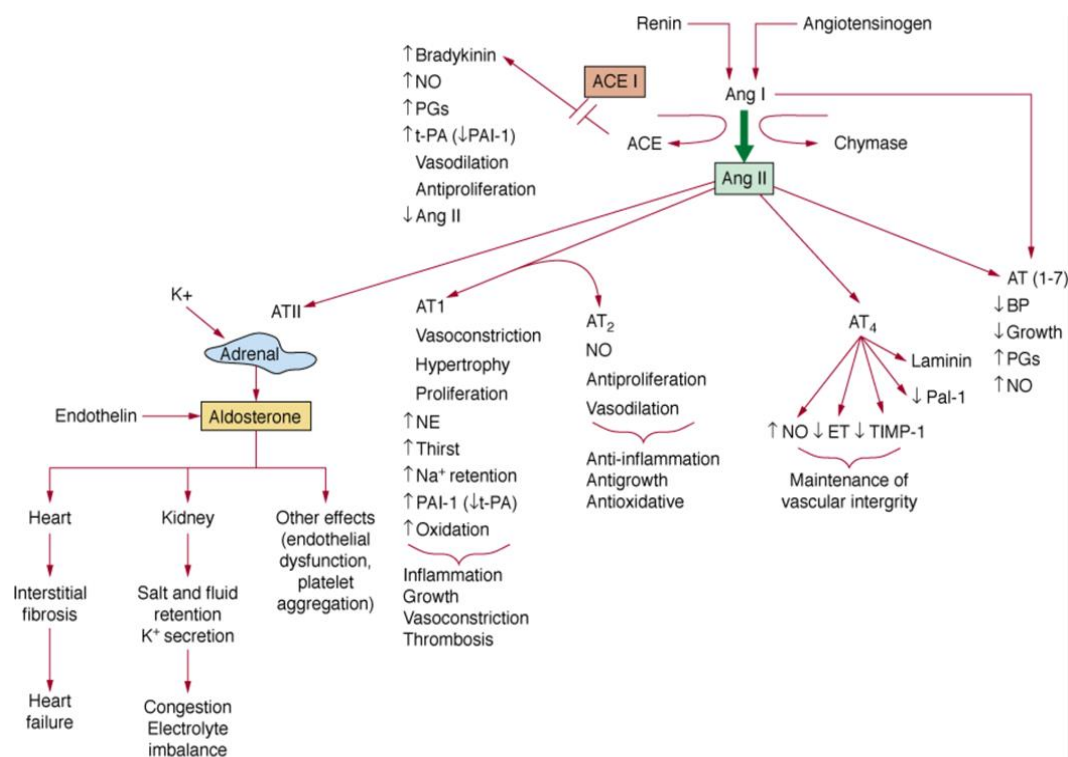
Within Minutes: RAAS

Renin is activated in the same timeframe as NOS as a response to a decrease in BP.

Renin is a protein synthesized in the kidney, brain, adrenal glands, visceral adipose tissue and vascular tissue [112]. It cleaves angiotensinogen to angiotensin I (Ang I), the rate limiting step in the RAAS pathway [113]. Ang I is hydrolyzed by angiotensin converting enzyme (ACE) to form angiotensin II (Ang II), which activates the vasoconstrictive effects in this pathway which is illustrated in Figure 7 [114].

When bound to angiotensin type I receptor (AT1), Ang II is a vasoconstrictor and serves as an inhibitor of further renin release [115]. Ang II also acts on the adrenal glands to release Aldosterone which causes the kidneys to reabsorb sodium [55]. Ang II also interacts with NADPH oxidase which can impair the cGMP signaling pathway initiated by NO [116]. Because this pathway has a powerful vasoconstrictive effect in vascular tissue, it has been highly studied for pharmacological treatment of elevated BP and HTN [117].

Despite the vasoconstrictive effects of Ang II, there is also evidence that Ang I can be cleaved to produce Ang (1,7) which causes vasodilation when paired with AT1 receptors [118, 119]. Ang (1,7) interacts with bradykinin to inhibit the activity of ACE and thus accentuate vasodilation [120, 121]. While many roles for this vasodilatory component of RAAS have been proposed, the function of this alternate pathway is not yet clear [122, 123].

Figure 7: Pathway of Renin-Angiotensin-Aldosterone System

Angiotensinogen is cleaved by Renin to form Ang I. Ang I is cleaved by ACE to form Ang II, which binds to AT1 to cause vasoconstriction [124]. Pairing of Ang II with AT2 and AT4 and formation of Ang II (1-7) are less studied aspects of RAAS and may have vasodilatory effects [110].

Study of the RAAS components is accessible through many methods. All of the proteins are relatively stable and so many enzyme-linked immunoassays are utilized to determine concentrations in blood and tissues [125]. Renin is sometimes quantified by plasma renin Activity (PRA) or the amount of Ang I produced during an incubation period [126], but immunoassays are more accurate at lower detection levels.

Within Hours: Renal fluid volume pressure control and capillary fluid shift

The ability of the body to conserve or excrete electrolytes also impacts intercellular and extracellular fluid balance [127]. The balance of solute that is restricted from cellular membrane transit (including sodium) between these two bodies of fluid creates a pressure gradient known as osmotic pressure. The flow of fluid across the membrane is influenced by electrolyte

concentrations and osmotic pressure [128]. As different BP regulatory systems alter electrolyte balance and blood volume, osmotic pressure changes and results in fluid shifts in the renal and capillary systems.

One hormone directly ties fluid shifts with BP regulation. Atrial natriuretic peptide (ANP) is a hormone, secreted by the atria of the heart, that aids in fluid and electrolyte balance to help regulate blood pressure [129]. In response to atrial dilation, ANP is secreted to inhibit renin secretion and aldosterone synthesis, thus preventing vasoconstriction and increasing osmotic pressure from sodium conservation [127]. These changes happen in a longer timeframe as opposed to the acute effectors described above.

Within Days: Aldosterone

Aldosterone synthesis is triggered through the action of Ang II and its primary role is to increase sodium reabsorption in the renal collecting ducts [36]. Aldosterone synthesis can also be initiated by acetylcholine, though it is not the primary regulator of this protein [130]. Reabsorption of sodium in the kidneys increases blood volume and results in BP increases [131]. The actions of aldosterone are slower than other BP regulatory systems and can have continued effects for days after an acute BP change [132]. Aldosterone may be measured in blood or in a 24-hour urine sample [133]. Aldosterone to Renin ratio is utilized to determine primary aldosteronism, a deficiency in aldosterone production [134]. Mineralocorticoid receptors mediate aldosterone's effect [135], and these are commonly a pharmacological target for BP regulation [136].

Study of Blood Pressure

While there is a greater understanding of BP in the twenty-first century than there was 100 years ago, it is still difficult to study given the vast array of influences. Guyton, Coleman and Granger made significant contributions to the field by creating a model of circulatory regulation

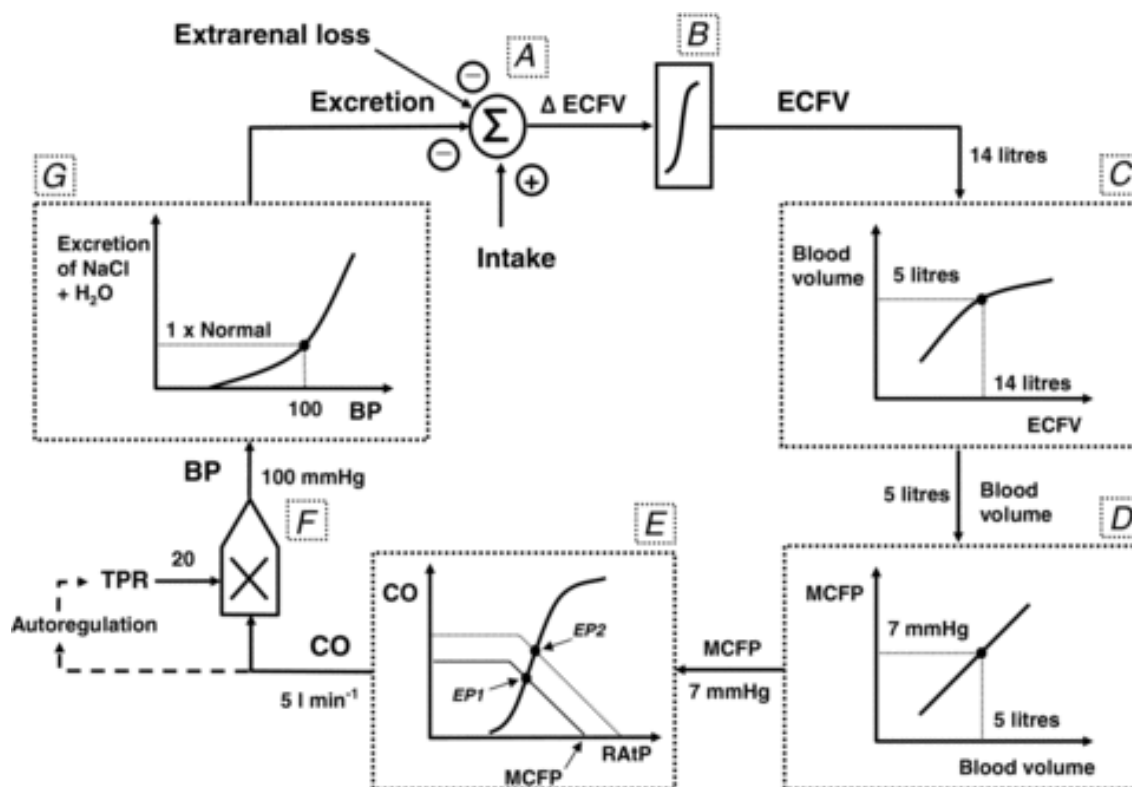
that offered a mathematical understanding of the relationship between the circulatory and renal systems [137]. Change in solute concentration influences blood volume, which in turn influences arterial pressure as shown in Figure 8 [138, 139].

BP in most studies and medical settings is measured utilizing a manual sphygmomanometer [140]. A BP cuff is placed around a patient's arm just above the elbow and a stethoscope is used to hear the heart beating at high and low pressure. The pressure is read by a gauge and corresponds with the systolic (high) and diastolic (low) BP [66]. Typically, pressure is read once after a patient sits in an upright rested position. Arm to arm differences in BP require that a dominant arm or the same arm be used for comparison purposes between BP measurements [141].

In some cases, BP measurement is taken multiple times with a 5 to 10 minute recovery window in between [142]. Studies using this method measure BP at one point in time or at multiple times across months or years [143, 144]. Data from hospital visits, doctors' offices and clinical studies are all utilized to consider BP change across lifetimes, between various individual characteristics and in a variety of environmental conditions [145].

Other studies utilize ambulatory BP monitors to record many measurements in a short period, which is much more accurate [146]. An average or range of BP is a key measurement drawn from these data [147]. Many studies consider changes in BP in relation to these factors over time [148], but fail to look at variability [30] or acute patterns. Very recently, reports of post hoc analysis of ambulatory BP studies indicate a link between BP variability and HTN and CVD [147, 149].

Figure 8: Guyton Model of Circulatory Regulation



“Any imbalance between intake and output of salt will lead to a cascade of events that oppose the initial disturbance, a classical negative feedback loop.” Change in extracellular fluid volume (ECFV) shown in boxes A, B and C affects mean circulatory filling pressure (MCFP) as indicated in box D and CO as indicated in box E. Changes in CO lead to autoregulation of solute concentration in the kidney shown in box F which will shift BP if sodium excretion is not increased as shown in box G [150].

Another common method to study BP is to look at changes in regulation of blood pressure and sodium processing. A 24-hour urine sodium collection offers insight to renal function [151]; paired with urine creatinine, it determines the effectiveness of the kidney in clearing sodium from the body [152]. Increased urinary sodium output is associated with higher BP in large population studies [153-156]. This method is one of the preferred means of assessing HTN risk [157, 158].

Sodium and Blood Pressure

Acute BP regulatory systems, NOS and RAAS, are both affected by sodium intake [64, 159, 160]. When pressure rises, sodium increases solute concentration. The resulting increase in blood volume can cause vessel distension and sheer stress, which can trigger NO production and blood vessel dilation [52, 161, 162]. When pressure decreases the RAAS cascade is activated [160, 163-165], and BP is increased by vasoconstriction initiated by Ang II and sodium conservation induced by aldosterone. If these two pathways are in check, BP remains within an individual's normal range with minimal fluctuation. However, like other homeostatic systems, high levels of stress for extended periods of time result in dysregulation [29].

Sodium is a critical mineral in the body for maintaining cellular balance, nervous system signaling, muscle contractions, glucose transport and many other functions [139, 166, 167]. A substantial portion of sodium is contained in bone [168], and there is evidence of an independent bone RAAS [169]. The normal range of sodium in the plasma is 137 to 142 mmol/L [170]. To maintain plasma sodium levels, as little as 391 mg of sodium is required daily from dietary sources [171]. If plasma sodium falls below this range, it is called hyponatremia and can result in neurological impairment and death [172]. Plasma sodium above this range is called hypernatremia and can result in neuromuscular complications, metabolic complications and renal failure [173, 174].

The World Health Organization recommends consuming less than 2g of sodium daily for optimal health [175]. However, sodium has historically been eaten in excess of 2.2 g per day [171]. The average daily sodium consumption in the US is 3.9g [176]. Globally it is 3.95g per day, ranging from 2.18 to 5.51g regionally [177]. High sodium diets are prevalent in countries that consume high quantities of processed foods [178]. In cross sectional studies, high sodium diet is

highly correlated with elevated BP and risk for HTN. The nature of this relationship remains unclear despite years of research in this area.

Efforts to reduce sodium in the diet have been in effect via national and world health guidelines for over 40 years [179]. During this time, many interventions have considered the effect of reduced sodium diet on BP, risk for HTN and risk for CVD [180, 181]. Some studies have found improvement following a low sodium diet, but not all [182-184]. Some individuals demonstrate a greater risk for elevated BP and HTN when eating a low sodium diet [185]. At a population-wide level, there is still a significant controversy regarding the benefit of reducing dietary sodium levels [177, 186, 187].

Insulin, a hormone primarily responsible for promoting glucose transport across cellular membranes, is also a key protein involved in sodium metabolism [188]. Increases in plasma insulin levels act on the kidney to stimulate sodium conservation [188]. In addition, sodium-glucose co-transporters have a key role in maintaining cellular glucose and sodium levels [189]. Because of this association, elevated BP is often a consequence of impaired glucose metabolism and vice versa [190, 191]. Individuals susceptible to hyperinsulinemia or diabetes are especially vulnerable to developing HTN [70, 188, 192].

Certain individual characteristics seem to make some people in a population more susceptible to developing HTN in response to high sodium diet. Sex has been noted as a factor in BP levels, with men tending to see greater effect from high sodium intake [193-195]. Individuals with an obese BMI are at greater risk for developing HTN. Also, those with diabetes are at greater risk, possibly due to an association with high levels of glucose [16, 196].

“Sodium sensitive” is the term used to describe individuals whose BP increases in response to a high sodium diet compared to a low sodium diet [197]. Reliability of this trait has not yet been proven [198]. However, it does support the idea that there are certain traits that

make individuals susceptible to developing HTN, and that not the entire population is at risk [199]. Identification of the key characteristics that influence BP is critical for early detection and treatment.

MTHFR and Blood Pressure

Several genes are associated with HTN including those altering kidney function, RAAS metabolism, and NO production. The relationship between genes involved in BP regulation and long term BP dysregulation is direct. There are many other genes associated with long term BP dysregulation in genome wide association studies (GWAS) that are part of unrelated metabolic systems [200, 201]. One gene, associated with elevated BP in GWAS codes for the enzyme MTHFR [202-206] (chemical structure in Appendix A1). This riboflavin-dependent enzyme is part of the one-carbon metabolism pathway which generates cofactors and methyl groups for nucleotide synthesis, amino acid metabolism, methylation reactions and protein synthesis [207, 208].

The primary role of MTHFR is to reduce methylenetetrahydrofolate (5,10-MTHF) to methyltetrahydrofolate (5'-MTHF). The main sources of 5,10-MTHF are dietary folate and folic acid [209]. Folic acid is the unsubstituted, fully oxidized, synthetic form of folate that is used in supplements and fortified foods [210]. Folic acid must be reduced to dihydrofolate and then tetrahydrofolate to become metabolically active [211]. This pathway is illustrated in figure 9 [212]. Homocysteine receives a methyl group from 5'-MTHF to become methionine. Methionine joins with an ATP molecule to become S-adenosyl-methionine (SAM), the primary donor of methyl groups to other molecules. S-adenosyl-homocysteine (SAH) is left after the methyl transfer and become homocysteine once again by shedding adenosine [213].

Two known single nucleotide polymorphisms (SNPs) in MTHFR, 1298A→C (rs1801131) and 677C→T (rs 1801133), are associated with elevated BP in GWAS [200, 202, 203, 214].

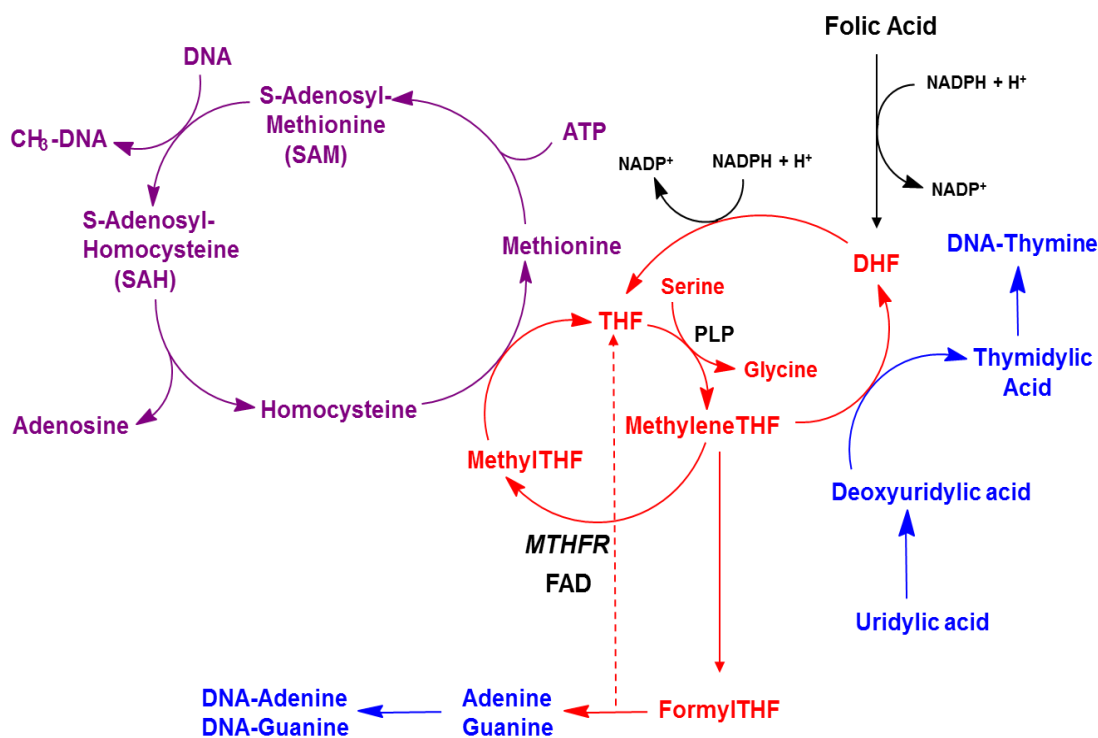
Prevalence of the 677TT homozygous variant differs among race/ethnicity groups from approximately 10% in Caucasian to 3% in African American and 18% in Hispanic populations and may be less in older populations [215]. The mutation causes a structural change in the folate binding site which lowers its affinity for its substrate, 5'-MTHF, and its cofactor, FAD [216]. Reduced activity of the MTHFR enzyme slows methyl donation to homocysteine for synthesis of methionine, thus causing accumulation of homocysteine in cells and subsequent export into the blood. Therefore, homocysteine levels increase when MTHFR is not efficient. Elevated homocysteine is associated with hypertension and cardiovascular disease [217, 218]. While it was thought that the link between MTHFR variants and BP was via hyperhomocysteinemia, intervention studies reducing plasma homocysteine levels failed to mitigate the effects of the MTHFR genotype on BP [219, 220].

In populations without mandatory folic acid food fortification, individuals with the MTHFR 677TT have BP equivalent to an 8 -10 years older individual with the wild-type genotype, and an average 6 mmHg greater than age-matched individuals with the MTHFR CC genotype [221]. When supplemented with folic acid, in an effort to drive the reaction and reduce homocysteine, blood pressure does not change [222]. When comparing CC and TT groups, the relationship between high BP and MTHFR genotype is greater in those with low plasma riboflavin levels [223]. Supplementation with riboflavin, the vitamin precursor for MTHFR's cofactor (FAD), causes blood pressure in TT individuals to decrease by as much as 6 mmHg post-intervention [224].

The MTHFR C677T polymorphism has been studied in relation to BP though there is still more knowledge needed to understand its role in the development of HTN. The impact of A1298C polymorphism is less understood, especially because the resulting variant enzyme is similar in nature to the wild type [225] and does not result in lower plasma folate levels or

higher plasma homocysteine levels [226, 227]. This mutation causes a substitution of alanine for glutamate in the enzyme and has a high association with cancer causing DNA methylation patterns [228, 229]. In vitro studies of enzyme activity found the enzyme with the 1298 substitution to have 68% of control activity while the 677 substituted enzyme had only 45% activity [230]. Prevalence of the 1298CC genotype also varies by race/ethnicity with roughly 5% in Chinese [231], 5% in Mexican/South American [232, 233], up to 30% in Caucasian [234] and 20% in African American [235] populations.

Figure 9: One Carbon Metabolism Cycle



Folic Acid is reduced by DHFR and MTHFR to produce 5'-MTHF which donates a methyl group to homocysteine for the formation of methionine. Modified from [212].

While both variants of the MTHFR enzyme are associated with higher disease risk, some studies suggest that heterozygosity for both variants is a greater risk factor than one or the

other [230, 236]. It is not possible for a fetus to survive with both variant alleles and these variants are trans to one another preventing a 1298AA/677CC pairing [237]. Plasma homocysteine levels have been highly correlated with individuals' combined genotype [238], especially with cardiovascular diseases [239].

Folate and Blood Pressure

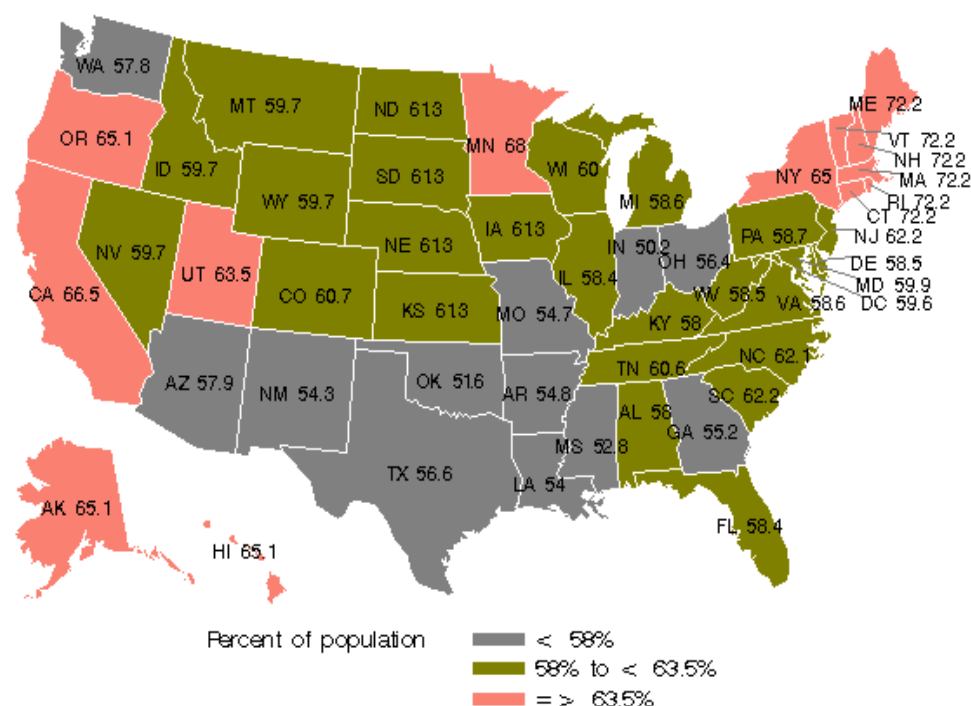
Folate (vitamin B-9) is a water-soluble vitamin that is not produced in mammals [240] (chemical structure in Appendix A1). Primary sources of folate for humans are supplements(usually in the form of synthetic folic acid), dairy products, dark leafy greens, beans, orange juice and some meats [241].Folate is required for the production of proteins, nucleotides and for amino acid metabolism [211]. Folates are reduced to provide methyl groups for these reactions through the one carbon metabolism cycle [242]. Folate is especially critical in early development [243, 244] and folate deficiency has significant clinical consequences, particularly for pregnant women [245-247]. The risk of neural tube defects is higher in folate deficient populations [248].

Figure 10 shows the percentage of individuals in the US with adequate folate intake, 400 dietary folate equivalent per day for adults [249]. More than half of surveyed individuals in the US have adequate folate intake. Women of childbearing age and non-hispanic black women are at greatest risk for deficiency [250].

Because of the significant risks associated with folate deficiency during fetal development, folic acid fortification of cereal grains is now common in many industrialized countries [251-255]. While this fortification has been successful at reducing the rate of neural tube defects [256], there is now question that fortification, particularly in combination with supplement use, is causing excessive intake in a significant percentage of the population [257].

Folates may be quantified by radioassay [258], microbiological assay [259] or liquid chromatography-mass spectrometry [260, 261]. These methods measure folate in red blood cells, plasma and serum [262]. The normal range of plasma folate levels is 4 – 20 ng and below this range is considered deficient [263, 264]. Maintenance of adequate tissue folate levels requires 100-200 ug dietary folate equivalents (DFE) per day taking into account only 25-50% bioavailability from foods and supplements [265]. To maintain optimal folate levels, adults 19+ years old are recommended to eat 400 ug DFE each day with an extra 200ug during pregnancy and 100 ug for lactating women [208].

Figure 10: Percentage of US Population with Adequate Folate Intake



Different colors represent different percentages of the adult population reporting intake of at least 400 dietary folate equivalent per day [249].

One way folate is potentially associated with BP is via homocysteine. When folate is deficient, homocysteine is not converted to methionine and concentration increases in the

plasma [266]. Therefore, homocysteine levels can be used as a marker of folate status [267]. Higher homocysteine levels are associated with elevated BP and with CVD [268, 269]. While the mechanism between the homocysteine-BP relationship is not known, interventions reducing homocysteine levels have failed to demonstrate an equivalent reduction in BP [219, 220].

Folate has also been associated with BP via 5'MTHF [210]. This reduced form of folate has been suggested to stabilize NOS allowing sufficient NO for vascular tissue dilation [270]. Supplementation with folic acid or 5-MTHF has reduced BP in some studies [271, 272], but the long term effects are not clear [273].

Riboflavin and Blood Pressure

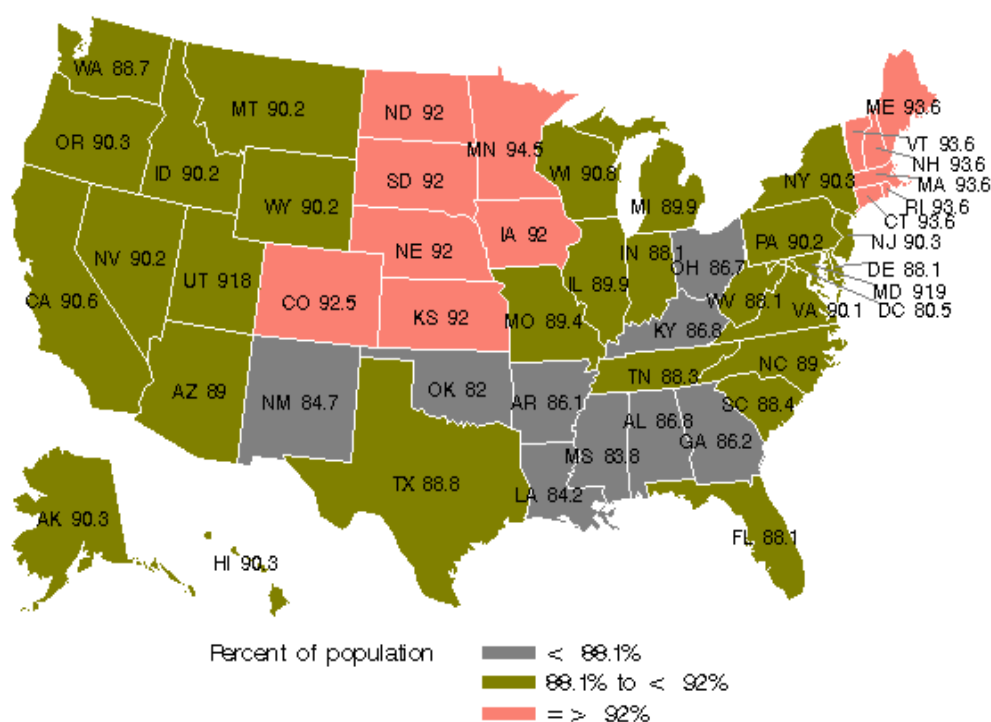
Riboflavin (vitamin B-2) is a water-soluble micronutrient important in humans as a precursor to two common enzymatic cofactors: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) [274] (chemical structure in Appendix A1). FAD and FMN have major functions in energy production, cellular and fat metabolism reactions [241]. Riboflavin is absorbed from diet and microbiome production mostly in the small intestine [275]. Minimal riboflavin is stored in the body, so a daily intake of 1.3 mg and 1.1 mg for adult males and females, respectively is recommended [241]. Dairy products, leafy greens, organ meats, some grains and supplements are rich sources of riboflavin [276].

Average riboflavin intake in the US is 2.5 mg in adult males and 1.8mg in adult women [277] and less than 6% of the population consumes less than the recommended dietary allowance [278]. Figure 11 illustrates riboflavin intake in the US population [279]. Sufficient plasma levels of riboflavin are from 6.2-39.0 nmol/L [280, 281]. Deficiency manifests in endocrine issues, vitamin malabsorption, liver and nervous issues [282]. Riboflavin is determined in red blood cells using the erythrocyte glutathione reductase activity assay (EGRac) [283]. This assay measures the ratio of activities of glutathione reductase, an enzyme that

requires FAD as a cofactor, with and without added FAD [284]. A ratio from 1.0 to 1.3 indicates an individual has sufficient FAD for the enzyme's activity; ratios above 1.3 indicate less than sufficient FAD levels [285].

Riboflavin is associated with BP primarily as a cofactor for enzymes related to BP regulation [83, 204]. As cited above, NOS is a key enzyme involved in BP regulation that requires both FAD and FMN as cofactors [99]. FAD is also a required cofactor for renase, which regulates sodium and phosphate excretion [286]. And most relevant to this thesis, MTHFR is an enzyme that has been associated with elevated BP and requires FAD as a co-factor [221, 222].

Figure 11: Percentage of US Population with Adequate Riboflavin Intake



Different colors represent different percentages of the adult population reporting intake of at least 1.8 mg riboflavin per day [279].

Role of MTHFR, Folate and Riboflavin in NO Production

MTHFR plays a critical role in the conversion of homocysteine to methionine by providing a methyl donor, 5'-MTHF. When this donor is not produced in high enough concentration, there becomes an excess of homocysteine in the cell [207]. Additionally, since homocysteine's precursor is SAH, there is more of this molecule present which inhibits SAM production and thus prevents methylation of DNA and other methyl-acceptors [287], see also Figure 9.

Increased levels of homocysteine in the plasma are associated with a number of cardiovascular conditions [288, 289]. Normal plasma levels range from 4-10 $\mu\text{mol/L}$ [290-292] and in diseased conditions it can be as high as 200 $\mu\text{mol/L}$ [268]. Above a healthy range, homocysteine can cause cellular damage, oxidative stress and increased excitotoxicity in certain neurons [293].

Individuals with the MTHFR 677TT gene variation have higher levels of homocysteine than their 677CC counterparts [294]. Aside from possible cellular damage caused by homocysteine, these elevated levels may be responsible for inhibiting NO production. Through inhibition of dimethylarginine dimethylaminohydrolase (DDHG), which breaks down nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA), higher levels of homocysteine lead to less nitric oxide production [295].

The association between high homocysteine levels and MTHFR677 genotype is modulated by an individuals' folate status [296]. Higher levels of folate can drive MTHFR's reaction to maintain a lower overall homocysteine level [297]. However, individuals with MTHFR TT genotype have lower levels of 5'-MTHF and NO without a change in homocysteine [298], suggesting more complexity to the relationship between MTHFR and NO. Individuals with polymorphisms in DHFR also lead to lower levels of 5'-MTHF and NO [299]. Supplementation of

5'-MTHF has a positive effect on NO production [300]. Current literature suggests 5'-MTHF serves a key role in NO production [301].

One connection between MTHFR and NO production is the NOS cofactor, BH₄. As previously stated, BH₄ stabilizes the NOS homodimer structure and this determines whether the enzyme is coupled, producing NO, or uncoupled, producing radical oxygen species [302]. Therefore BH₄ synthesis inhibitors are also NO synthesis inhibitors [303]. BH₄ is available either through conversion from BH₂ or de novo synthesis from sepiaterin [97]. BH₂ acts as a competitive binder to NOS preventing electron transfer across the homodimer and causing uncoupling [304, 305].

DHFR is one enzyme that serves to recycle BH₄ from BH₂ [306, 307], and limited activity of DHFR leads to low levels of NO [308]. While increasing folate levels (via folic acid supplements) may serve to lower homocysteine levels, high levels of intracellular unmetabolized folic acid may cause competition in the substrate binding site of DHFR which would limit recycling of BH₄ [97, 309]. The MTHFR binding site is a similar structure to DHFR and competition for its binding site may also limit recycling of BH₄ [301].

Gaps in Literature

The body of knowledge surrounding BP regulation, micronutrient intake and genetic risk factors is broad. However, the ability to predict an individual's risk for developing HTN is limited to existing knowledge and there are holes. First, BP is primarily studied in a resting state, i.e. a single point measurement of a dynamic system. This leaves out information about what changes during homeostatic regulation. Given the intricate control of BP and its relationship to pulmonary and renal systems, a measurement that captures as much of this information as possible is necessary. Information about how BP responds directly following salt ingestion may

provide a clearer picture of the mechanism causing increases in BP in relationship to sodium intake.

Second, it is not clear how genes indirectly related to BP regulation, like MTHFR, are risk factors for development of HTN. Elevated BP can be reduced in MTHFR TTs with riboflavin supplementation. The relationship between this gene and riboflavin and how it impacts BP is not understood. Additionally, MTHFR and BP have only minimally been studied and in few populations. To know if this is an effective global intervention and to understand how this gene and riboflavin affect BP, further study of these effects in other populations is critical.

CHAPTER 1 OF THE DISSERTATION: Aims and Hypothesis

Overall, the aim of this project is to establish physiological patterns of acute blood pressure (BP) homeostasis that may relate to an individual's risk for HTN.

Specific Aims

Aim 1: Determine the degree to which ingestion of a sodium load impacts acute BP response to test the hypothesis that BP fluctuates within minutes of sodium ingestion to regulate blood pressure. This aim will determine whether acute BP response to oral sodium ingestion exists.

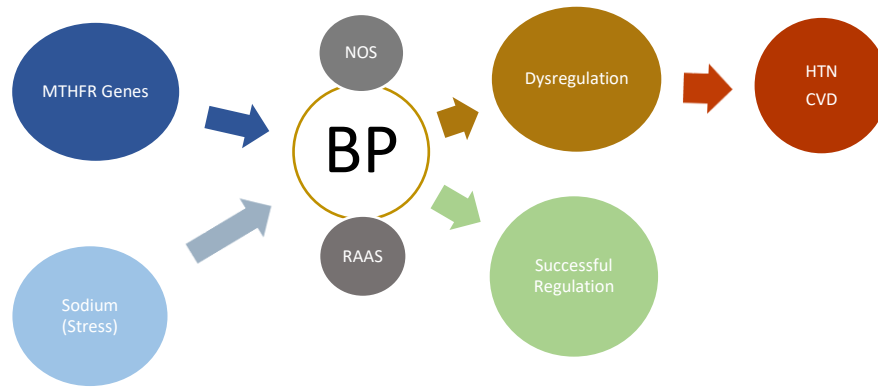
Aim 2: Determine the association between MTHFR genotype, riboflavin status and BP to test the hypothesis that resting BP is correlated with MTHFR genotype and that riboflavin status influences this correlation. This aim serves to validate previous literature on the relationship between BP, riboflavin and MTHFR genotype in a folate replete population.

Aim 3: Assess the impact of MTHFR genotype on acute BP regulation following sodium ingestion to test the hypothesis that an individual's MTHFR genotype and riboflavin status will impact the acute BP response. This aim seeks to demonstrate the influence of MTHFR on the acute BP response and suggest a mechanism via NOS.

Innovation

The proposed work takes a novel look at BP regulation via individual acute BP response. In addition, these studies examine acute BP changes in response to genes and diet and compare this measure of BP with resting BP.

Figure 12: Model of Dissertation Hypothesis



This model is the framework for this work. Dietary and genetic inputs impact BP. BP is regulated by NOS and RAAS pathways via these inputs. The result is regulation or dysregulation, the latter leading to HTN and/or CVD.

CHAPTER 2 OF THE DISSERTATION: An Acute Blood Pressure Response to Salt Ingestion in Healthy Subjects

Introduction

The number of individuals globally with high blood pressure (BP) and related complications is increasing [310, 311]. The World Health Organization suggests a link between dietary sodium intake and development of hypertension (HTN) [175]. The recommendation to prevent HTN and cardiovascular disease (CVD) is to maintain a low sodium diet, less than 2000 mg sodium per day [312]. While this reduced sodium diet has been recommended in the US since 1980 [18], a recent meta-analysis of randomized controlled trials for dietary sodium reduction concluded no reduction in risk for HTN or CVD [20]. This evidence about the lack of effect of dietary sodium reduction adds to the confusion about the associations between sodium and increasing BP and how to prevent resulting complications.

BP is closely regulated in the body via constriction and dilation of the vessels supplying blood to the body, controlled primarily by the sympathetic nervous system and the endocrine system [313]. In the short term, renin or nitric oxide (NO) are produced to maintain a constant pressure [132]. In the long term, these measures are complemented by the action of the kidney filtering or secreting sodium and potassium to manage blood volume [314]. Many forms of HTN result from dysfunctions in the kidney that prevent efficient mineral and volume regulation [56]. A high sodium diet has a clear impact on a system impaired in its ability to regulate this mineral. Yet, essential HTN is the form that is most commonly diagnosed globally and treated poorly for lack of understanding in how it manifests [315].

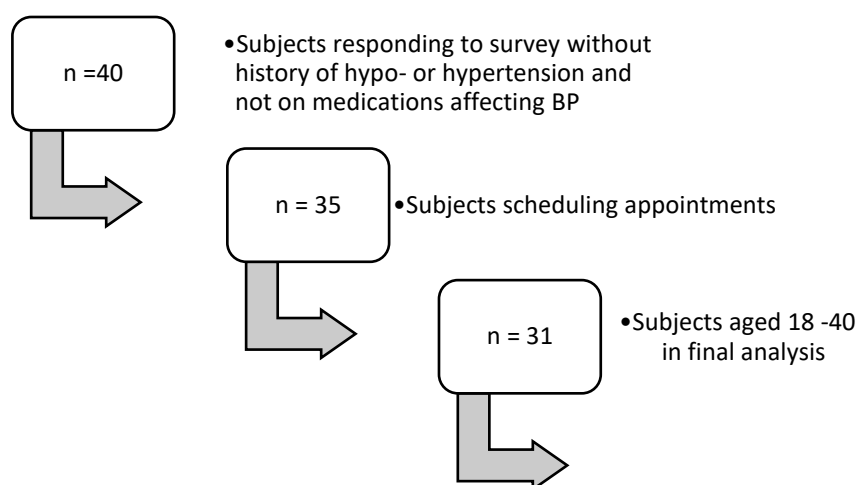
Sodium has a dilatory effect on vagal tissue resulting from vascular sheering and release of NO. The ability of the vessels to dilate is dependent on health of this reflex and plasticity of

the tissues. The result of diminishing effectiveness of this regulation is clear in increasing BP over time [200]. In the short term, blood pressure fluctuates as is noted in ambulatory BP readings [316]. While studies have considered the long-term effect of a high sodium diet [160, 317, 318], there is no documented evidence in humans of the acute response to sodium intake. In this study, we investigated the changes in BP immediately following ingestion of an oral sodium load. We hypothesized that BP would demonstrate different patterns following ingestion of an oral sodium load compared with a water alone.

Methods

Subjects: Thirty-five individuals aged 18 to 40, of forty screened, agreed to participate in the study; of these thirty-one completed the study as shown in Figure 13. To recruit subjects, we posted signs around Rutgers campus and sent emails to previous lab subjects. Prior to beginning the study, instructions for testing procedures were sent electronically and subjects who agreed to participate signed consent forms. All subjects received payment at the completion of the study. The study was approved by the Rutgers University Office of Research Regulatory Affairs Institutional Review Board.

Figure 13: Acute BP Study Recruitment and Retention Data



Blood Pressure Protocol: Subjects arrived at the lab fasted (only water allowed) for a minimum of six hours and sat for 10 minutes to allow a resting pattern to begin. A Littman® blood pressure cuff was placed just above the elbow of a relaxed, straight arm and two resting readings were taken, 10 min apart. After another 10 minutes, participants drank the test or control solution. Readings were taken at 10, 20, 30, 40, 50 and 60 minutes post ingestion. Throughout the testing session, subjects remained in a seated, resting state with the same posture. For each subject, changes in mood, physiological state and cravings at each time point were recorded (see form in Appendix A4); these responses were open-ended but the following examples guided the participants: mood (happy/sad), physiology (tired/anxious/hungry), cravings (food/drink). Subjects returned for 6 total sessions, with at least forty-eight hours in between sessions, to complete 3 trials per stimuli.

Stimuli: Solutions were prepared within 1 week of use using NaCl (99%, Sigma S5886) and Millipore deionized water. The water solution was 475 mL of deionized water and the salt solution was 475 mL of 157 mM NaCl (1.9 g sodium) in deionized water. All solutions were warmed to 30 C prior to subject ingestion.

Questionnaire: Upon beginning the study, subjects completed a diet and lifestyle questionnaire (see form in Appendix A5) to ascertain their typical sodium intake and factors that may affect their blood pressure. Subjects provided liking, quantity and frequency of intake for foods typically high in salt such as cheese, table salt, and canned soups. The questionnaire also asked basic health information including frequency of exercise, drug use and previous hyper- or hypotension diagnosis. No subjects were excluded; statistical allowances accounted for any factors affecting BP.

Data Analysis: We recorded all measures in Excel and used this data analysis tool as well as Stata. For each subject, we averaged data from all trials for each stimuli for systolic (SBP) and

diastolic (DBP) pressures. Resting BP for each subject was calculated by averaging the first two readings (prior to ingestion) of all 6 trials combined. Two-way ANOVA was used to determine differences between group response to each stimuli. Regression analysis was conducted to look for relationships between subject characteristics and BP responses. Findings were considered significant if $p < 0.05$.

Results

Participant Characteristics: Participants, age 18 to 40 years, were healthy, without pre-existing hypertension diagnosis. Asian, African American and Caucasian individuals participated. No participants used medication that are known to affect BP (2 subjects reported use of birth control and 1 a vitamin supplement).

Average age of subjects was 23.5 ± 4.0 . Average BMI was 23.6 ± 4.4 . Resting SBP of subjects averaged 106.6 ± 10.8 mmHg and was consistent across trials. There were minimal differences between males and females in the study; no significant differences are noted amongst BMI, age or resting BP (Table 2).

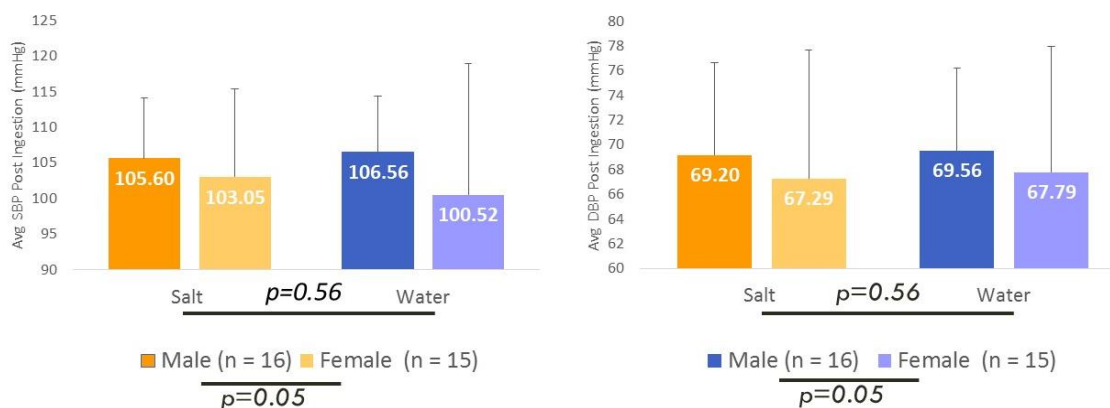
	n	BMI	Age (years)	Resting Systolic BP (mmHg)	Resting Diastolic BP (mmHg)	Ethnicity			
						African	Asian	Caucasian	Hispanic
M	16	24.7 ± 4.3	22.2 ± 5.42	108.8 ± 9.3	70.5 ± 8.4	1	9	6	0
F	15	24.2 ± 4.6	24.2 ± 5.0	104.0 ± 10.8	66.3 ± 9.7	1	6	6	2
TOTAL	31	23.6 ± 4.4	23.3 ± 5.2	106.6 ± 10.8	68.4 ± 9.2	2	15	12	2

Table 2: Acute BP Study Subject Characteristics

Blood Pressure Responses: Overall average SBP during the experimental period was similar following ingestion of water compared with salt ($p = 0.68$), (Figure 14). Male subject average SBP (salt = 106.8 ± 8.6 ; water = 106.7 ± 9.2 mmHg) post ingestion was 5.06 mmHg greater ($p = 0.11$) than female average SBP (salt = 102.3 ± 11.9 mmHg; water = 101.13 ± 17.8 mmHg). Overall

average DBP during post ingestion was also comparable following both stimuli ($p = 0.56$). Male subject average DBP (salt = 70.9 ± 6.6 ; water = 70.8 ± 6.7 mmHg) post ingestion was 5.6 mmHg greater than female average DBP (salt = 64.9 ± 9.7 ; water = 65.7 ± 9.4 mmHg) ($p = 0.05$).

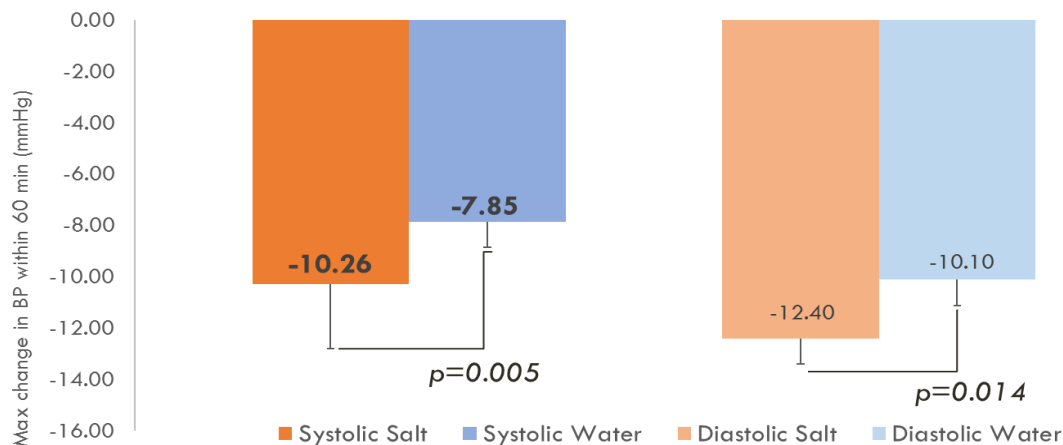
Figure 14: Acute BP Study Average SBP and DBP Post Salt Ingestion



Average of all subjects average SBP (left) and DBP (right) during 60 minutes following ingestion. Males (darker bars) and females (lighter bars) following either a salt (orange bars) or water (blue bars) bolus.

Though the average BP over 60 minutes post salt or water did not differ from baseline or between salt and water, transient fluctuations in BP were observed in each subject. These included decreases in BP that occurred at different times for different subjects. The maximum

Figure 15: Acute BP Study Change in SBP and DBP Post Salt Ingestion

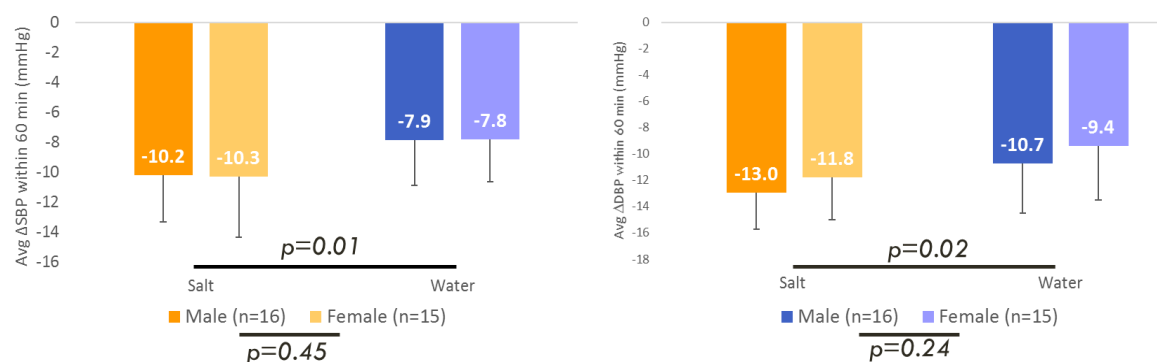


Salt ingestion (orange bars) compared with water ingestion (blue bars) in Systolic (left bars) and diastolic (right bars) pressure. Salt minus water ingestion SBP = -2.33 ± 3.78 , DBP = -2.22 ± 4.72

decrease in SBP following sodium ingestion was greater than the maximum decrease following water ingestion for the group ($p=0.005$) (Figure 15). The same was observed for DBP ($p=0.014$). Decrease in DBP was larger than the decrease in SBP following both water and sodium ingestion.

Though SBP and DBP in female subjects decreased slightly less than in male subjects (SBP difference = 0.06 mmHg, DBP difference = 1.27 mmHg), differences in response between males and females were not significant (SBP, $p=0.45$; DBP, $p=0.24$). Both males and females demonstrated a greater decrease in pressure following salt ingestion ($p < 0.02$) (Figure 16).

Figure 16: Acute BP Study Change in SBP and DBP post Salt Ingestion by Sex



Average of all subjects change in SBP (left) and DBP (right) during 60 minutes following ingestion. Males (darker bars) and females (lighter bars) following either a salt (orange bars) or water (blue bars) bolus.

Questionnaire Results: Participants reporting eating approximately 23.8 – 131.2 g of sodium in a month from common food sources including potato chips, bread, table salt and cheese. The most commonly reported sodium containing food items were cheese, bread and cereal. Subjects reported eating foods high in sodium between 8 and 190 times per month. Differences in salt intake between males (1.8 ± 0.7 g/day) and females (1.9 ± 0.9 g/day) were minimal: $p = 0.3$. Frequency of consuming high salt foods was similar between males (2.2 ± 0.7 times/day) and females (2.1 ± 1.1 times/day): $p = 0.39$. BP was not associated with estimated monthly intake or frequency of eating sodium. The strongest associations were between average DBP and SBP

post salt ingestion (Table 3) and estimated frequency of high sodium food intake (DBP $R^2 = 0.34$; $p = 0.01$ and SBP $R^2 = 0.27$; $p = 0.03$).

	ESTIMATED [NA ⁺] INTAKE (G)		EST [NA ⁺]-RICH FOOD FREQUENCY	
	R^2	p	R^2	p
RESTING SBP	0.03	0.10	0.21	0.07
RESTING DBP	0.01	0.08	0.13	0.05
AVG SBP POST SALT	0.05	0.05	0.27	0.03
AVG SBP POST WATER	0.02	0.06	0.12	0.07
AVG DBP POST SALT	0.08	0.01	0.34	0.01
AVG DBP POST WATER	0.02	0.07	0.19	0.05
Δ SBP POST SALT	0.04	0.04	0.20	0.05
Δ SBP POST WATER	0.04	0.01	0.02	0.00
Δ DBP POST SALT	0.04	0.03	0.01	0.95
Δ DBP POST WATER	0.04	0.01	0.06	0.75

Table 3: Acute BP Study Associations between BP and Dietary Sodium

Discussion

The mechanisms that cause high dietary sodium intake to lead to hypertension are still not well understood. We sought to understand how sodium immediately impacts BP levels by measuring BP during a period acutely following ingestion of salt. Our data indicate that BP decreases immediately in response to either salt or water ingestion, but more so in response to salt. This is consistent in a small group of thirty-one subjects and suggests that BP is regulated closely in response to sodium intake.

The BP response found in our study may indicate a reflex to sodium ingestion. Sodium ingestion increases blood sodium concentration within 15 minutes in animals [319, 320] and certain mechanisms respond to maintain extracellular ion concentration [321]. In vitro studies of animal tissue demonstrate a relaxation of vascular tone within minutes of gavage with sodium solution [317, 322, 323]. Additionally, studies on rat muscle provide suggestion of a reflex related to sodium concentration – a contraction in response to low levels followed by

relaxation once homeostasis is reached [324]. Relaxation of vascular tone causes decreases in BP [325] and would explain the immediate decrease in BP seen in our cohort. The possibility that it is a reflex and that homeostatic mechanisms are at work would explain why BP in this cohort returned to a baseline shortly after the initial decrease occurred.

In Guyton's circulatory model, changes in blood volume are directly related to changes in arterial pressure [150]. Since blood volume is directly impacted by fluid and sodium ingestion [69, 326], the impact of the bolus ingested by our subjects would be reflected in their BP. In this study, we did not measure blood volume or blood sodium levels. However, in another study, increases in blood sodium related to changes in BP within 120 minutes following a sodium infusion [327]. The BP effects seen in this study are likely reflective of homeostatic blood volume and blood sodium changes immediately following ingestion. The understanding of this reflex and how it changes over time, may provide insight to how hypertension develops in seemingly healthy individuals.

A great deal of literature suggests that certain individuals are salt sensitive [328] and these individuals are more susceptible to developing hypertension. In one study, this trait was reproduced at separate points in time and demonstrated that salt sensitive individuals had a greater increase in BP with age compared to non-salt sensitive individuals [329]. The acute BP response was seen in our entire cohort, but no characteristic seemed to suggest different responses that would indicate salt sensitivity. It is possible that a larger cohort of subjects is required to see this differentiation.

Many cohort studies demonstrate differences in BP related to sex [193], with men being at greater risk for developing HTN [194]. In this study, we found minor differences in average BP, but not in the effect of sodium ingestion. This may suggest that absolute BP levels are

different between males and females, yet the reflexes related to sodium ingestion are similar in healthy subjects.

Estimated dietary sodium intake was correlated with average SBP and average DBP post sodium ingestion in this study. The conclusions that can be drawn from our questionnaire are limited since self-reported dietary intake data has high variability of accuracy [330] and our cohort size is small. The relationship seen in our data shows that greater dietary sodium intake is related to higher average BP following ingestion. This study's subjects were healthy, and average SBP and DBP post sodium ingestion was not associated with resting SBP or DBP. Over a period of years, it may be possible to determine if this higher average SBP and DBP post sodium ingestion correlates with higher resting BP.

Summary

In this study, we demonstrated that there is an acute transient decrease in BP following a sodium ingestion that is similar in healthy men and women. This decrease may be related to immediate changes in blood sodium and blood volume that are part of a homeostatic reflex. Understanding this reflex and its efficiency over time may shed light on the development of essential hypertension. Additionally, understanding the BP effect of sodium ingestion may serve as a tool in cases where lower BP is desired, but drug interactions prevent full use of hypovolemic or anesthetic drugs [331].

CHAPTER 3 OF THE DISSERTATION: An Anticipatory Blood Pressure Response to Salt Taste in Healthy Subjects

Introduction

Just as feeding begins well before food enters the mouth through sight, smell and neural signaling, digestion also begins prior to food entering the stomach via anticipatory responses [332]. Simply tasting a sweet substance triggers the release of insulin in anticipation of forthcoming sugars [333, 334]. This early release of insulin aids in maintaining homeostasis by preparing the body for incoming nutrients [335]. Anticipatory effects triggered by taste are documented in animals and humans for glucose [336, 337] and amino acids [338]. Signals initiate from taste receptors on the tongue unique to different taste compounds: sweet, salty, bitter, umami and sour [333, 339, 340]. It is known that epithelial sodium channels (ENaC) in the tongue are part of the signal cascade to the sensory cortex recognizing salty taste [341, 342] and that they are involved in renal handling in rats [336]. As sodium concentrations in the body are closely regulated, like glucose concentrations, it stands to reason that an anticipatory effect exists for salt taste.

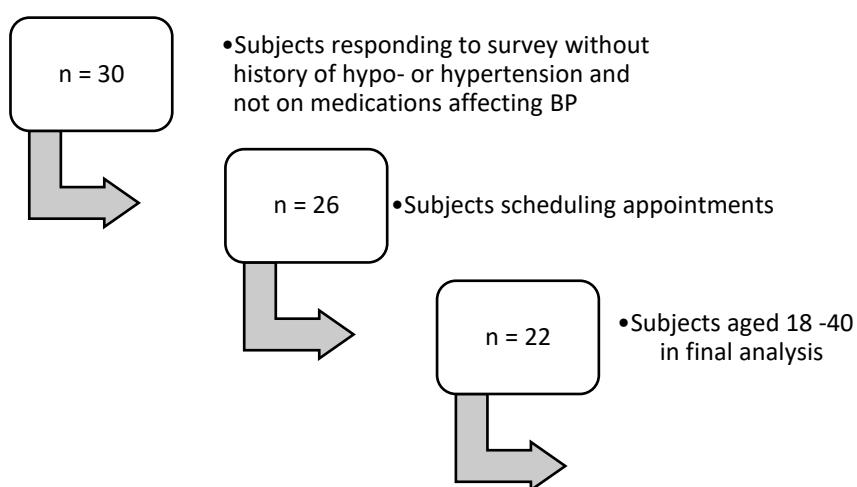
Sodium homeostasis is regulated in the short term via the renin-angiotensin-aldosterone system (RAAS) [164] and via nitric oxide synthase (NOS) [161]. In the long term, it is the kidney that primarily regulates sodium balance [56, 64]. One of the changes that occurs in response to changing sodium balance is blood pressure (BP) [132]. The study of dietary salt intake and BP has spanned decades and provided little mechanistic evidence for the correlations between chronic high sodium intake and hypertension (HTN) [317]. Not only is hypertension one of the 15 leading causes of death in the US, it is strongly correlated with risk for stroke and cardiovascular disease [175].

The majority of diagnosed HTN is essential [7], without a known cause, and is difficult to treat [196]. In recent studies, the cephalic phase response to sugars has been used to assist in regulating blood glucose levels who are prone to dysregulation, such as diabetics [343]. In previous studies, we demonstrated that ingestion of salt causes an acute decrease in BP. In this study, we sought to identify an anticipatory BP effect related to salt taste.

Methods

Subjects: Twenty-two individuals, aged 20 to 30, agreed to participate in the study. To recruit subjects, we posted signs around Rutgers campus and sent emails to previous lab subjects. We accepted subjects who committed to the length and protocol of the study and who had no prior history of smoking, BP related conditions or regular chewing gum use (which may affect taste perception). Prior to beginning the study, instructions for testing procedures were sent electronically and subjects signed IRB approved consent forms. All subjects received payment at the completion of the study. The study was approved by the Rutgers University Office of Research Regulatory Affairs Institutional Review Board.

Figure 17: Anticipatory Response Study Recruitment and Retention Data



Taste and Blood Pressure: Subjects arrived at the lab fasted (no food or drink) a minimum of six hours and sat for 5 minutes to allow a resting pattern to begin. A Littman® BP cuff was placed just above the elbow of a relaxed, straight arm and a reading taken. After one resting blood

pressure reading (-10 min), just prior to the next reading, participants rinsed and spit 4 times, 30 seconds each time, with 40 mL of 1.0 M sodium chloride (NaCl) solution or deionized water (Millipore). Readings continued for every 10 minutes 2 hours following for a total of 14 readings during 130 minutes. Throughout the testing session, subjects remained in a seated, resting state with the same posture. We instructed subjects to record their mood, physiological state and cravings at each time point (see form in Appendix A4); these responses were open-ended but the following examples guided the participants: mood (happy/sad), physiology (tired/anxious/hungry), cravings (food/drink). Participants returned at the same time at least two days per week for multiple weeks to complete five trials with each solution.

Questionnaire: Upon beginning the study, subjects completed a diet and lifestyle questionnaire to ascertain their typical sodium intake and factors that may affect their BP (see form in Appendix A5). Subjects provided liking, quantity and frequency of intake for foods typically high in salt such as cheese, table salt, and canned soups. The questionnaire also asked basic health information including frequency of exercise, drug use and previous BP diagnosis of hypo- or hyper-tension. No subjects were excluded; statistical allowances accounted for any factors affecting BP.

Data Analysis: We recorded all measures in excel and used this data analysis tool package as well as Statistica. For each subject, we averaged data across the trials for each condition for systolic (SBP) and diastolic (DBP). Resting BP for each individual is an average from the first reading (prior to rinsing) of all the trials. T-test of paired means and two-way ANOVA were used to calculate differences between stimuli and group comparisons. Significance was considered $p < 0.05$.

Results

Participant Characteristics: Participants, age 18 to 29, were healthy, without pre-existing hypo- or hypertension diagnosis. Asian, African American, Caucasian and Hispanic individuals participated (Table 4). No participants used medications that affect BP or taste perception (2 subjects reported use of birth control). In total, 14 females and 8 males with an average age of 23.5 years old, completed the study. The average was different between males, 26.6 ± 4.1 , and females, 21.7 ± 1.7 ($p = 0.001$). Resting SBP for the whole group was 110.0 ± 8.2 mmHg, and did not differ between females, 108.8 ± 5.9 mmHg, and males, 114.4 ± 10.4 mmHg ($p = 0.13$). Resting DBP for the whole group was 71.3 ± 6.6 mmHg, and was also similar between females, 69.9 ± 4.8 mmHg, and males, 73.6 ± 8.7 mmHg ($p = 0.22$).

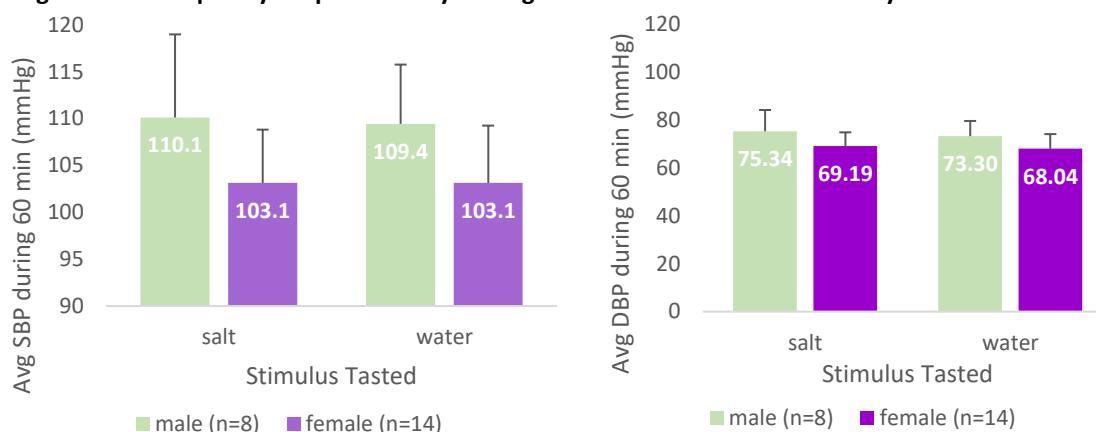
	n	BMI	Age (years)	Resting Systolic BP (mmHg)	Resting Diastolic BP (mmHg)	Ethnicity			
						African	Asian	Caucasian	Hispanic
M	8	26.6 ± 4.1	22.4 ± 3.1	117.7 ± 7.4	75.8 ± 7.4	1	5	3	0
F	14	21.7 ± 1.7	23.2 ± 2.9	106.3 ± 5.3	67.9 ± 4.0	0	4	6	2
TOTAL	22	23.5 ± 4.0	23.3 ± 3.7	110.9 ± 8.2	71.3 ± 6.6	1	9	9	2
p =		0.001	0.56	0.12	0.22				

Table 4: Anticipatory Response Study Subject Characteristics

Average BP Responses: The average of all BP readings following stimulus rinse was calculated for each subject and then averaged within the group. For all subjects combined, average SBP during the 110 minutes post rinse was not statistically different between a water rinse, 106.18 ± 7.1 mmHg and salt rinse, 106.3 ± 7.8 mmHg ($p=0.9$). Average DBP was also similar following a salt rinse, 71.8 ± 7.5 mmHg, and following a water rinse, 70.38 ± 6.75 mmHg ($p=0.5$). The group

average SBP following a salt and water rinse was 3.8 mmHg less than the group average resting BP.

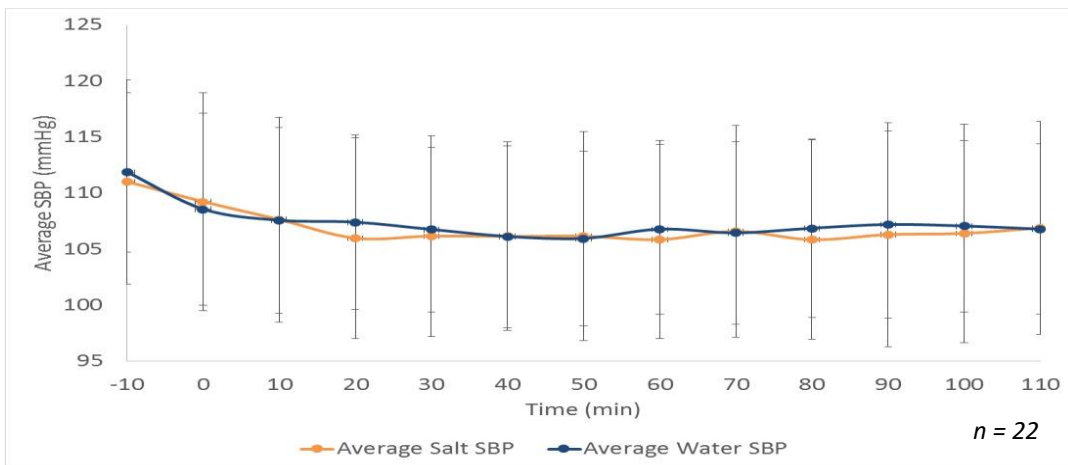
Figure 18: Anticipatory Response Study Average SBP and DBP Post Salt Rinse by Sex



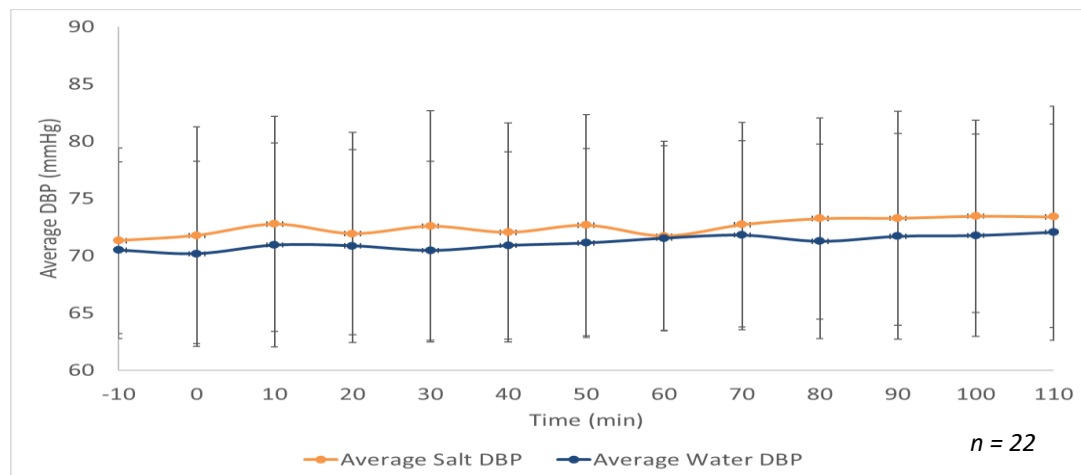
Average SBP (left) and average DBP (right). Male (green bars) and female (purple bars) following salt rinse (left two bars) and water rinse (right two bars).

Because there have been noted sex differences in BP, we calculated averages based on male and female groups (Figure 18). Males and female subjects average SBP ($p = 0.003$) and DBP (0.01) post rinse were unique, despite non-statistically different resting SBP and DBP. There were not statistical differences within sex groups in average SBP or DBP following a salt or water rinse ($p > 0.6$).

Group BP Response Patterns: To look for patterns in BP response, we graphed the average of all subjects BP at each time point following both a salt and water rinse (Figures 19 and 20). There is not a significant difference in the overall group average SBP ($p = 0.5$) or DBP ($p = 0.4$) pattern following a taste of water versus salt.

Figure 19: Anticipatory Response Study Average SBP Pattern Post Salt Rinse

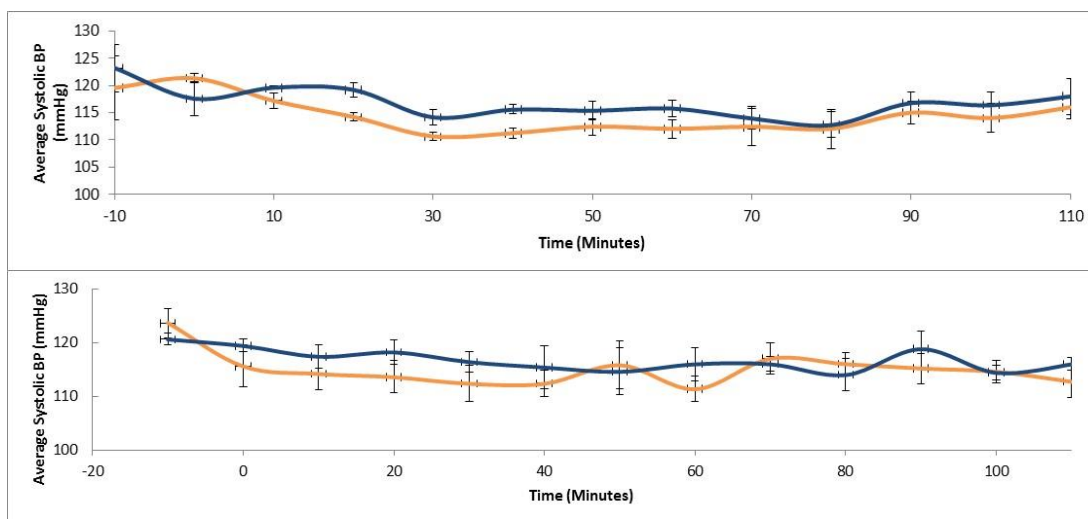
Group average pattern in SBP following salt (orange line) or water (blue line) rinse.

Figure 20: Anticipatory Response Study Average DBP Pattern Post Salt Rinse

Group average pattern in DBP following salt (orange line) or water (blue line) rinse.

Individual BP Response Patterns: Despite the lack of difference in the group average SBP and DBP and group BP response patterns, we noted transient decreases in all subjects, on average within the first 60 minutes, that differed following a salt and water rinse (Figure 21). However, the timepoint of the max decrease in BP varied between subjects. Because of these individual time differences, the group average response pattern is accounting for one subject's decrease in BP at the same time another subject may have an increase in BP following a decrease.

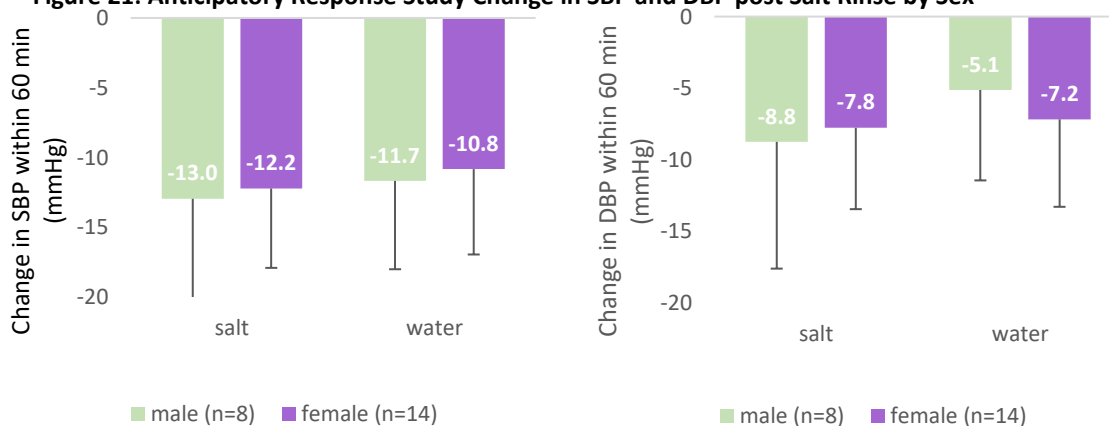
Figure 22: Anticipatory Response Study Example Individual SBP Pattern Post Salt Rinse



Individual SBP pattern (average of 3 trials per stimuli) following salt (orange lines) and water (blue lines) rinse. Female subject (top) and male subject (bottom).

Overall Change in BP: To make group comparisons of the individual differences observed in BP patterns, we determined each subject's average max decrease in SBP and DBP post rinse of water or salt (averaged by 3 trials per stimuli) and averaged those as a group. Group max decrease in SBP was on average 1.33 mmHg greater following salt rinse compared to water rinse ($p = 0.03$) (Figure 22). Differences between males and females were statistically different as well, 0.8 mmHg greater decrease in males compared to females ($p = 0.05$). Group max decrease in

Figure 21: Anticipatory Response Study Change in SBP and DBP post Salt Rinse by Sex



Average change in SBP (left) and average DBP (right). Male (green bars) and female (purple bars) following salt rinse (left two bars) and water rinse (right two bars).

DBP was 1.81 mmHg greater following salt rinse compared to water rinse ($p=0.04$). Change in DBP was also statistically different between males and females ($p = 0.05$).

Salt Diet Questionnaire Results: We collected information on typical intake of foods high in salt to understand if salt intake had a possible effect on BP response. Participants indicated using products commonly high in sodium between 30 and 182 times per month (Table 5).

	% Responding "Like"	Avg Times Consumed Per Month			% Responding "Like"	Avg Times Consumed Per Month
Potato Chips	93%	4.7		Pasta Sauce	78%	3.4
Pickles	48%	1.8		Cold Cereal	85%	12.1
Pretzels	63%	2.6		Beef Jerky	52%	1.2
Canned Soup	33%	1.6		Ketchup	78%	7.6
Table Salt	67%	18.1		Salad Dressing	63%	4.9
Cheese	89%	13.0		Pizza	89%	3.7
TV Dinners	19%	1.0		Cafes/Muffins	96%	5.2
				Total		80.9

Table 5: Anticipatory Response Questionnaire Diet Data

We correlated monthly frequency of salt intake with average resting SBP and DBP, average SBP and DBP following a salt and water rinse, change in SBP and DBP following a water and a salt rinse. Of these, the strongest significant correlations were with average DBP post water rinse ($R^2 = 0.20$, $p = 0.05$) and with average change in DBP following a water rinse ($R^2 = 0.40$, $p = 0.003$).

BP is based on daily rhythms so we asked participants for their normal waking time and correlated it with SBP and DBP and found no R^2 values greater than 0.10. Participants reported exercising 2.6 ± 1.5 times per week with no difference between male and female responses, and there were no correlations between times per week and resting SBP or DBP.

Discussion

Anticipatory responses serve many purposes in the body, including preparing the system for incoming metabolites[344]. Thus far, these responses are recorded for nutritive [334, 345] and toxic [346] molecules. We investigated BP responses to salt and water rinses to determine if an anticipatory response to salt exists. In this study, a group average decrease in BP was observed following a rinse with water or salt solution. Because BP decreased in relation to both water and salt, and both would affect fluid balance, this response could be related to blood volume changes [132].

Epithelial sodium channels (ENaC) have been associated with salt taste perception [347, 348], and there is evidence for a water “taste” that is perceived independent of other stimuli [349, 350]. It is possible that the use of water as a solvent for salt caused similarity in responses. However, there was decrease in BP following a salt rinse greater than following a water rinse.

Resting BP did not differ by sex; however, the average BP post rinse and the max decrease in BP post rinse was greater in males compared with females. Sex differences in BP regulation [193], estrogen levels [194] and responsiveness to orthostatic challenge [195] may contribute to these differences. Different taste perception between males and females may also contribute to the sex differences in this cohort [351-354].

We did not see associations between resting SBP or DBP with reported dietary salt intake. While there are several studies that do associate dietary sodium intake with long term BP changes [200, 329, 355], it is not clear how sodium intake in the short term leads to these outcomes. Our data did indicate that frequency of sodium food intake was associated with DBP post water rinse, but not post salt rinse. It is possible that those who ate salty foods more frequently found water to be an aversive stimulus [356-360], resulting in association with DBP.

Summary

In this study, transient decreasing BP patterns were observed in response to salt and water taste. Within the cohort, we observed a greater max decrease in BP following a salt rinse compared to a water rinse. These data suggest an anticipatory response that may be related to salt ingestion and BP regulation.

CHAPTER 4 OF THE DISSERTATION: MTHFR Genotype, Riboflavin and Resting Blood Pressure in Healthy Subjects

Introduction

High blood pressure (BP) is a leading cause of death globally [25]. Both genetic and environmental factors lead to elevated BP in one's lifetime. There are many genes considered to be risk factors for developing hypertension (HTN), some directly involved in BP regulation and others not. One important gene whose SNP variants are implicated in many age-related diseases is methylenetetrahydrofolate reductase (MTHFR) [361]. This gene plays a role in the reduction of methylenetetrahydrofolate ($\text{CH}_2=\text{THF}$ or 5,10-MTHF) to methyltetrahydrofolate ($\text{CH}_3\text{-THF}$ or 5'-MTHF). This reduced form of folate serves as a methyl donor to form methionine from homocysteine in the one carbon metabolism cycle which contributes to DNA methylation, nucleotide synthesis and other methylation reactions [362].

A polymorphism of the MTHFR gene, 677C→T, leads to an enzyme variant with lower affinity for its substrate, $\text{CH}_3\text{-THF}$, and its cofactor, flavin adenine dinucleotide (FAD). The result is a genotype associated with hyperhomocysteinemia [363], cardiovascular disease [364], Alzheimer's disease [365, 366] and elevated blood pressure [367]. The variant genotype (677TT) is present in about 15% of the US population, with higher prevalence in Hispanic populations [368, 369] and lower in African American ones [216].

Because the MTHFR enzyme is FAD-dependent, the association between BP and the 677C→T polymorphism is modulated by an individual's riboflavin status; those with the TT variant and low riboflavin levels have higher BP than those homozygous for the CC wild-type variant [223]. Supplementation with riboflavin reduces BP in individuals with this polymorphism

[224]. Studies in Northern Ireland confirm the impact of a riboflavin intervention in lowering BP in individuals with the variant genotype [221].

Given the prevalence of the variant and the lack of information about riboflavin levels in the US population, the need for information about the MTHFR-BP-riboflavin relationship is pertinent. In addition, it is not known if folic acid fortification in a population's food supply will affect the previously demonstrated BP-MTHFR association. We sought to determine the riboflavin status and BP levels of a healthy US subpopulation, exposed to folic acid fortification. We expected that folic acid fortification would not influence the relationship and that we would see higher BP levels in individuals with low riboflavin status and the TT genotype.

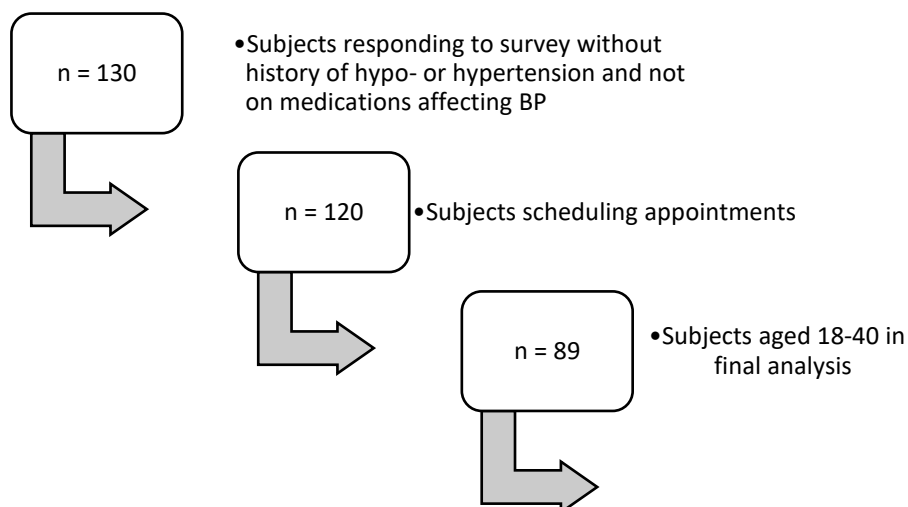
Methods

Subjects: A total of 130 subjects were recruited via flyer distribution across Rutgers campuses (Figure 23). Subjects were screened via phone or email and were excluded if they were not willing and able to provide a blood sample and if they were not between 18 and 40 years old. A total of 89 subjects, aged 18 to 40 participated in all aspects of the study including BP measurement, a diet and health questionnaire and blood collection. Prior to participation, subjects read and signed a consent form. After the study, subjects received a morning snack and compensation for their participation. The study was approved by the Rutgers University Office of Research Regulatory Affairs Institutional Review Board.

Experimental Protocol: Subjects arrived fasted for at least 8 hours for an appointment between the hours of 8am and 10am. BP was measured via manual sphygmomanometer and stethoscope reading on the left arm after a seated rest period of 10 minutes. BP measurement was recorded three times with a one-minute rest in between readings; an average of these readings was used for data analysis. Following this, subjects were asked a series of questions

about their medical history, medications and supplements taken, and their diet in the days prior to the appointment (see form in Appendix A6).

Figure 23: MTHFR Resting BP Cohort Study Recruitment and Retention Data



Blood collection and processing: A certified phlebotomist drew 15 mL of blood into an EDTA tube and 7 mL of blood into a serum separator tube via butterfly needle. Following collection, serum remained at room temperature for 30 minutes until centrifugation; and then the serum layer was removed into aliquots for freezing. EDTA plasma tubes were held on ice until centrifugation, followed by removal of plasma and buffy coat and washing of red blood cells. All samples were stored at -80°C until analysis.

Blood Analysis: MTHFR C677T genotype was determined via RT-PCR using a Taqman Sample to SNP kit and SNP assay (RS: 1801133). Riboflavin levels in the plasma were quantified via HPLC based on previously published methods [370, 371]. Riboflavin status was also determined via erythrocyte glutathione reductase activity (EGRac) in red blood cells via methods previously published [372]. Homocysteine was quantified via HPLC following methods previously published [373]. Total nitrate/nitrites were determined as a marker of NOS activity via a Caymen LDH immunofluorescent kit.

Statistics: Using Stata, data were analyzed for interactions among BP, MTHFR 677 genotype and riboflavin status. Additionally, data were analyzed for associations between calculated dietary intake of folate and riboflavin and measured blood values of these vitamins. All analyses were performed via multiple regression and ANOVA. To determine sample size, a power calculation was performed as follows: : Based on an alpha of 0.01, a power of 0.9, and a prevalence of the MTHFR 677TT homozygous genotype of 15%, 86 subjects were required to detect an estimated effect size of 1.2 mmHg difference in systolic blood pressure between MTHFR 677TT and 677CC/677CT subjects. Statistical significance in analysis was considered $p < 0.05$.

Results

Study Sample Characteristics: A total of 120 subjects were screened for the study. Of those, 89 were between the ages of 18 and 40 with an average age of 23.8 ± 5.5 and average BMI of 24.3 ± 5.1 (Table 6). There were 42 CCs, 33 CTs and 14 TTs demonstrating a 16% prevalence of the TT genotype in this study sample. The overall average SBP was 113.6 ± 11.6 mmHg and the average DBP was 72.8 ± 10.8 mmHg. There was a total of 40 males and 49 females. Most of the study

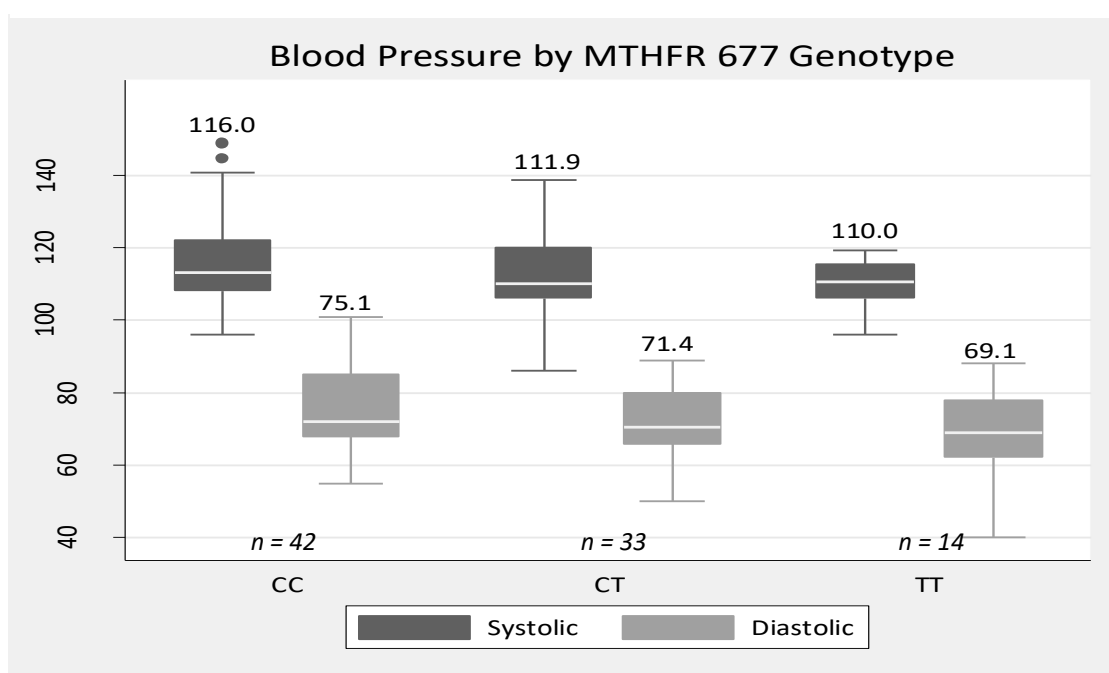
	n	BMI	Age (years)	Avg SBP (mmHg)	Avg DBP (mmHg)	Sex		Ethnicity			
						Male	Female	African	Asian	Caucasian	Hispanic
CC	42	22.5 ± 6.1	24.8 ± 5.2	116.0 ± 12.6	75.1 ± 10.9	19	23	8	10	21	3
CT	33	25.1 ± 4.4	24.0 ± 4.3	111.9 ± 11.7	71.4 ± 9.6	15	18	0	7	21	5
TT	14	24.2 ± 5.6	24.6 ± 6.5	110.0 ± 6.4	69.1 ± 11.7	6	8	0	4	9	1
TOTAL	89	23.8 ± 5.5	24.3 ± 5.1	113.5 ± 11.6	72.8 ± 10.8	40	49	8	21	50	9
p =		0.13	0.89	0.15	0.13	0.99					

Table 6: MTHFR Resting BP Cohort Study Subject Characteristics

population was Caucasian and Asian with some African American CCs and a range of Hispanic subjects in all three genotypes.

Blood Pressure: The average BP of all subjects was in a healthy range, less than 120 mmHg SBP and less than 80 mmHg DBP. Individuals with CC genotype had higher SBP and DBP than individuals with the CT or TT genotype, but the difference was not statistically significant. Between CCs and TTs there was a 3.8 mmHg difference in both SBP ($p = 0.15$) and DBP ($p=0.13$) (Figure 24).

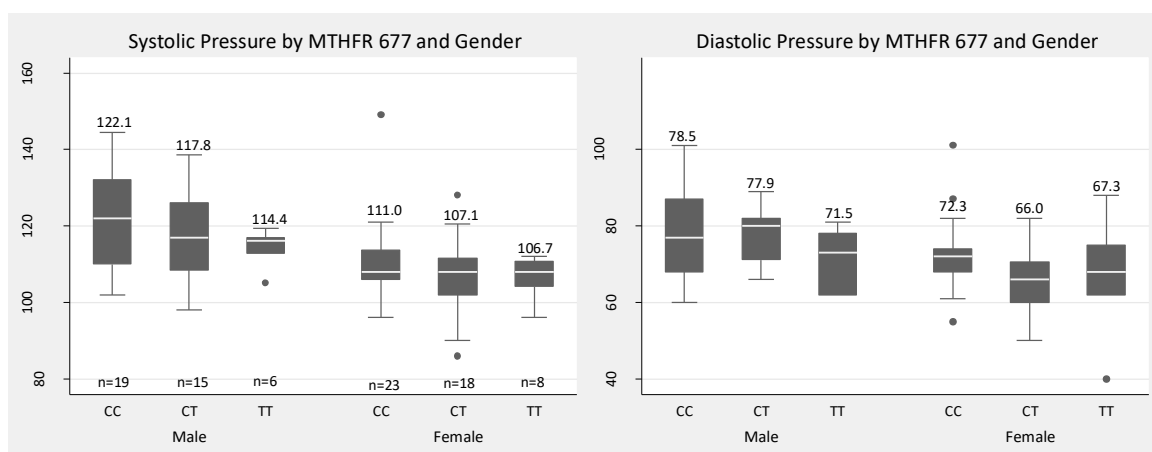
Figure 24: MTHFR Resting BP Cohort Study Average SBP and DBP by Genotype



Average SBP (dark grey) and DBP (light grey) did not differ between genotypes

($p = 0.13$ and $p=0.15$, respectively)

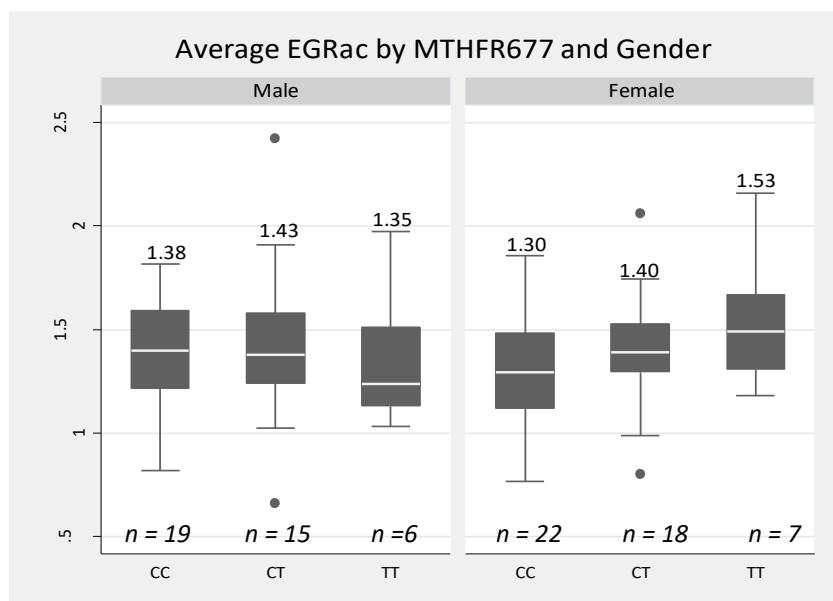
Though there was not a difference between BP across MTHFR genotype, average SBP and DBP was higher in males than in females for all genotypes (Figure 25). Overall male SBP was 119.3 ± 11.3 mmHg and overall female SBP was 108.9 ± 9.7 mmHg ($p < 0.01$). Overall male DBP was 77.2 ± 1.6 mmHg and overall female DBP was 69.2 ± 1.4 mmHg ($p < 0.01$).

Figure 26: MTHFR Resting BP Cohort Study Average SBP and DBP by Genotype and Sex

There is no difference between MTHFR genotypes ($p=0.1$), but BP differed between males and females within genotypes ($p<0.01$).

EGRac and BP: Using EGRac as an indicator of riboflavin status, we categorized individuals by high (1.3 and above) and low (below 1.3) [374]. High EGRac ratio indicates that an individual is deficient in riboflavin. Average EGRac ratio was slightly higher in CTs (1.4 ± 0.3) and TTs (1.46 ± 0.3) than in CCs ($1.3 \pm$

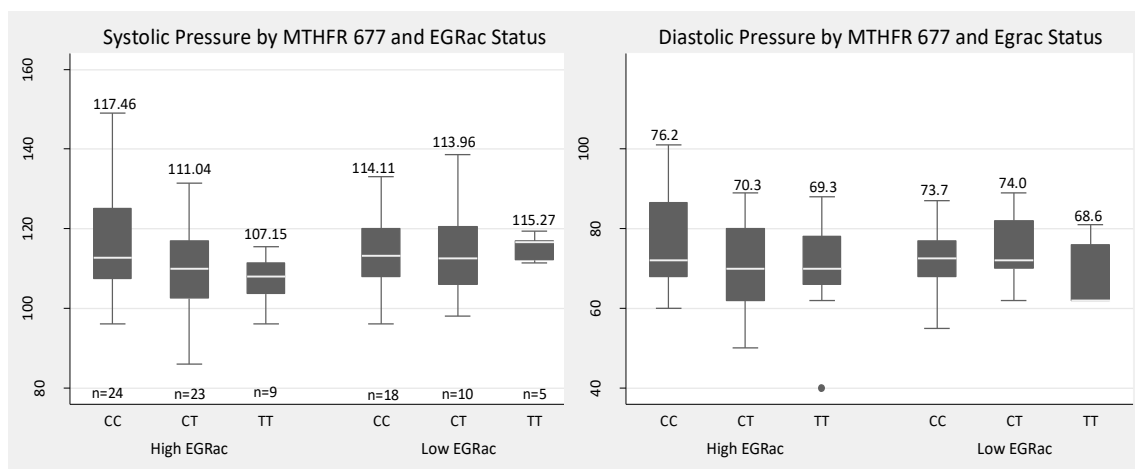
0.2), but not significantly different ($p=0.32$). We considered there may be a sex difference among the genotype groups; however, EGRac was similar across genotypes in males ($p=0.8$) and females ($p=0.1$) (Figure 26).

Figure 25: MTHFR Resting BP Cohort Study EGRac by Genotype and Sex

EGRac ratio above 1.3 indicates riboflavin deficiency. No difference between MTHFR groups in females ($p=0.1$) or males ($p=0.8$).

To determine if riboflavin status impacted BP, we compared BP across genotypes split by high and low EGRac ratio (Figure 27). SBP was not different between CCs ($p = 0.4$) and CTs ($p = 0.515$) in either the high or low EGRac groups; however, TTs with high a EGRac ratio had a lower SBP than those with low EGRac ratio. ($p = 0.014$). DBP was not different across genotype groups when compared by high or low EGRac ratio (CC, $p = 0.46$; CT, $p = 0.33$; TTs, $p=0.92$)

Figure 27: MTHFR Resting BP Cohort Study Average SBP and DBP by Genotype and EGRac

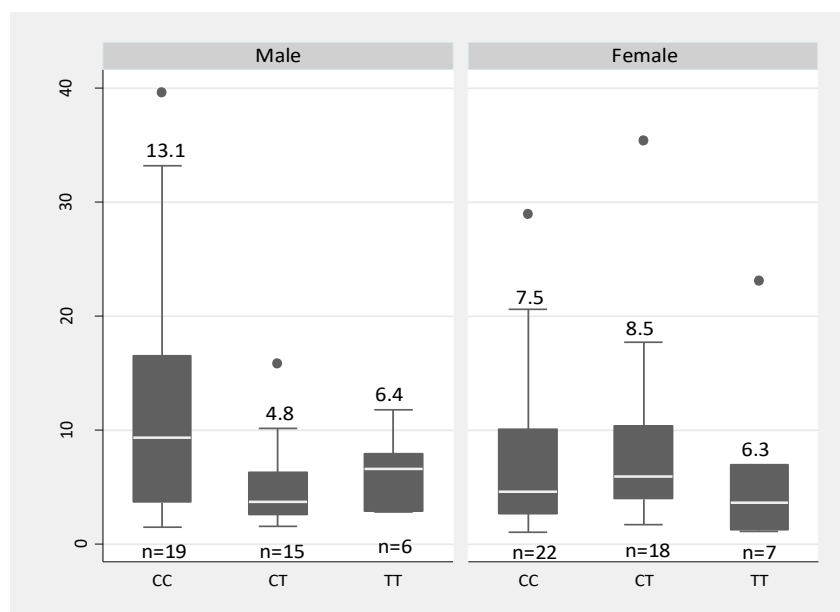


Average SBP was different between TTs with high or low EGRac, but DBP was not different in any group.

Plasma Riboflavin:

EGRac has traditionally been used as a measure of riboflavin status; we measured plasma riboflavin to determine its relationship with BP and with EGRac in this cohort. Overall plasma riboflavin was $10.1 \pm$

Figure 28: MTHFR Resting BP Cohort Study Plasma Riboflavin by Genotype and Sex



Plasma riboflavin differed across genotypes in males ($p=0.02$) but not in females ($p=0.8$)

9.9 $\mu\text{mol/L}$ in CCs, $6.8 \pm 6.6 \mu\text{mol/L}$ in CTs and $6.3 \pm 5.9 \mu\text{mol/L}$ in TTs, not statistically different ($p=0.15$). Given the BP differences between males and females, we considered sex differences in plasma riboflavin and found greater values in CCs than CTs and TTs in males ($p=0.02$), but no differences in females ($p=0.8$) (Figure 28).

Despite the difference in plasma riboflavin across genotypes seen in males, plasma riboflavin was not associated with SBP in males ($p=0.6$) or females ($p=0.09$). We considered an interaction between MTHFR genotype and riboflavin and did not find evidence in this cohort ($p=0.72$). DBP was also not associated with plasma riboflavin in males ($p=0.6$) or females ($p=0.6$), and an interaction between MTHFR 677 and riboflavin was not a predictor of DBP ($p=0.4$).

Since EGRac ratio is meant as a predictor of riboflavin status, we looked for a correlation with plasma riboflavin. Despite the fundamental relationship between these measures, EGRac and plasma riboflavin values were not correlated ($R^2 = 0.02$, $p=0.18$).

Figure 29: MTHFR Resting BP Cohort Study Dietary Riboflavin Intake by Genotype and Sex

Dietary Riboflavin

and Folate Intakes:

Dietary riboflavin

and folate intake

data were

collected to

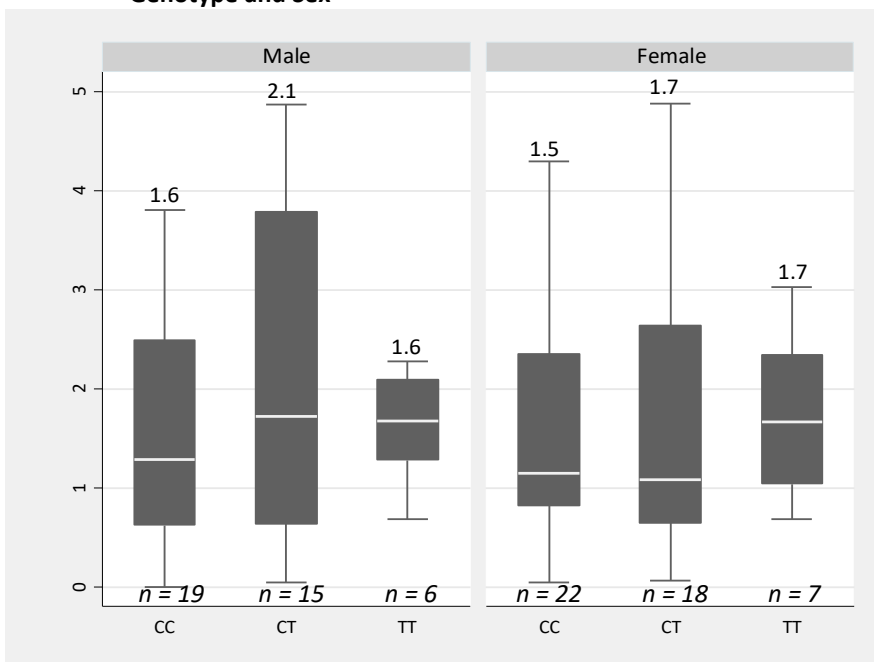
determine if

individuals had

high or low

consumption that

may affect status.



There were no significant differences in dietary riboflavin intake across genotypes ($p=0.48$); nor across genotypes in males ($p=0.5$) and females ($p=0.8$), separately.

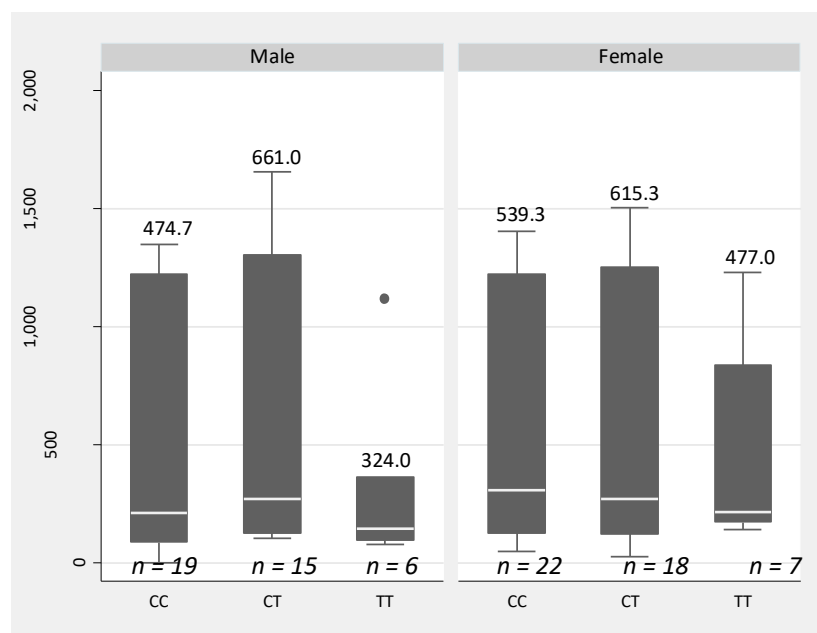
The recommended dietary intakes of riboflavin are 1.3 mg for adult males and 1.1 mg for adult females [241]. Average daily intakes in the NHANES III survey were 2.5mg for males and 1.8 mg for females [277]. Average intakes of the subjects in this study were above the recommended dietary intake levels and close to the NHANES average dietary intake. There were no significant differences in dietary riboflavin intake across genotypes ($p=0.48$) (Figure 29). Differences across genotypes between males ($p=0.5$) and females ($p=0.8$) were also not significant.

Neither average SBP ($p=0.15$) nor DBP ($p=0.07$) was associated with dietary riboflavin intake. When MTHFR and dietary riboflavin values were compared to SBP and DBP, no associations were found, nor was there a significant interaction ($p=0.3$). Dietary intake of riboflavin did not predict plasma riboflavin levels ($p=0.2$) or EGRac ($p=0.1$).

Recommended dietary intake of folate in the US is 400 ug dietary folate equivalent (1ug DFE = 1 ug food folate) for both males and females [375]. Average daily intake per the NHANES III Survey is 454 to

652 ug DFE [250]. Average dietary folate intake in this cohort was above recommended dietary intake in all groups except for male TTs (Figure 30). There was not a significant difference in dietary folate intake across

Figure 30: MTHFR Resting BP Cohort Study Dietary Folate Intake by Genotype and Sex



No differences in dietary folate were present between MTHFR genotypes ($p=0.36$) or between males and females ($p=0.76$)

genotypes ($p=0.36$). Neither average SBP nor DBP was significantly associated with dietary folate intake ($p=0.2$ and $p=0.8$, respectively).

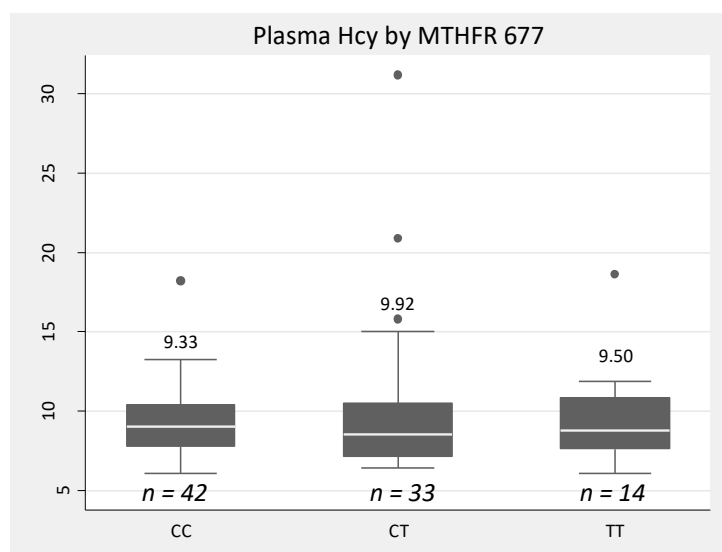
Homocysteine: Elevated

homocysteine levels have previously been associated with HTN and with the MTHFR 677TT genotype. To determine if homocysteine differed between genotypes in this cohort, we measured plasma levels. Normal plasma Hcy levels are 4-10 $\mu\text{mol/L}$ and values above 12-15

$\mu\text{mol/L}$ are indicative of hyperhomocysteinemia [287]. Average homocysteine level for this cohort was within the normal range, $9.6 \pm 3.6 \mu\text{mol/L}$. Homocysteine levels were similar among the genotypes: CC = $9.3 \pm 2.3 \mu\text{mol/L}$; CT = $9.9 \pm 4.9 \mu\text{mol/L}$; TT = $9.5 \pm 3.1 \mu\text{mol/L}$ ($p=0.8$) (Figure 27). Average levels of plasma Hcy differed between males ($11.0 \pm 4.6 \mu\text{mol/L}$) and females ($8.7 \pm 2.4 \mu\text{mol/L}$) ($p=0.002$) (Figure 31).

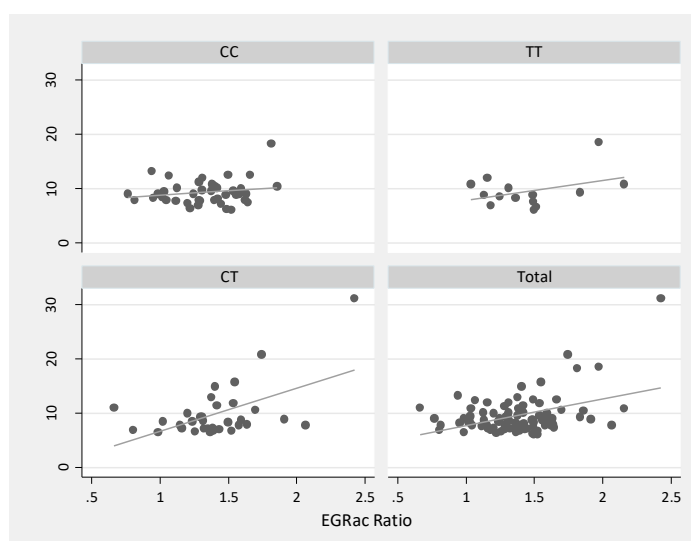
Interestingly, EGRac was directly associated with plasma

Figure 31: MTHFR Resting BP Cohort Study Plasma Homocysteine by Genotype



There is no difference between genotypes in plasma Hcy ($p = 0.8$)

Figure 32: MTHFR Resting BP Cohort Study Relationship between EGRac and Homocysteine

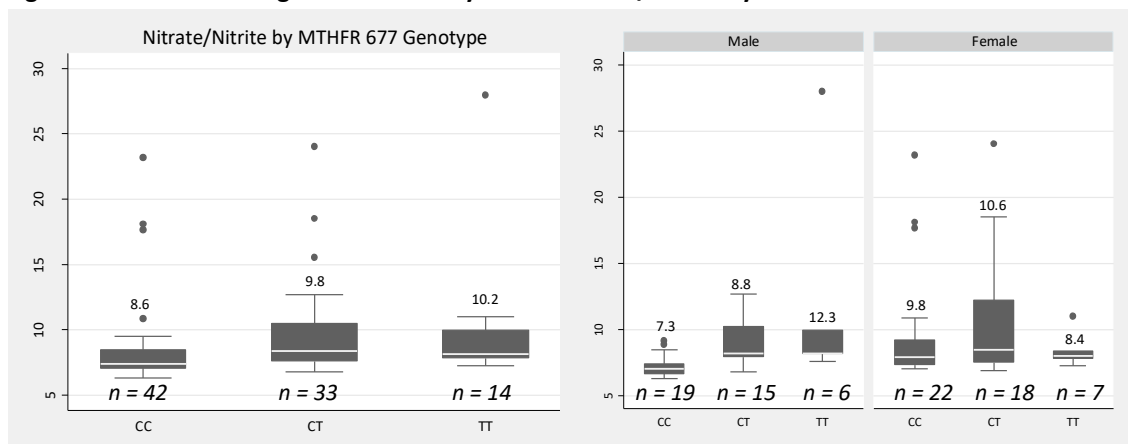


EGRac ratio and genotype are predictors of plasma homocysteine

homocysteine levels ($p=6 \times 10^{-4}$) and there was an interaction between MTHFR genotype and EGRac ($p=0.05$). The association was strongest in the CT genotype group ($R^2=0.3$, $p=0.001$) and not significant in the CC ($R^2=0.04$, $p=0.2$) or TT ($R^2=0.14$, $p=0.2$) genotype groups (Figure 32). Plasma homocysteine did not predict SBP ($p=0.1$) on its own, but it was a predictor ($p=0.05$) after controlling for MTHFR genotype in a multiple regression analysis. DBP was not significantly associated with plasma homocysteine alone ($p=0.7$) or when controlling for MTHFR genotype ($p=0.4$).

Plasma Nitrite/Nitrate: Seeking additional information about the relationship between MTHFR and BP, we measured nitrites/nitrates as an indicator of NO production. Higher levels of NO should correspond with lower BP. In this cohort, nitrates/nitrates in plasma were not significantly different among the MTHFR genotypes in the whole cohort ($p=0.32$) (Figure 33, left); however, plasma levels were different across genotype in males: CC = 7.3 ± 0.9 umol/L; CT = 8.8 ± 1.6 umol/L; TT = 12.4 ± 8.8 umol/L ($p=0.01$) (Figure 33, right). The difference across genotypes was not present in females in this cohort: CC = 9.8 ± 4.5 umol/L; CT = 10.6 ± 4.6 umol/L; TT = 8.4 ± 1.3 umol/L ($p=0.5$).

Figure 33: MTHFR Resting BP Cohort Study Plasma Nitrate/Nitrite by Riboflavin and EGRac



Comparing plasma nitrite/nitrate within genotypes did not show differences ($p=0.32$). Comparing plasma nitrite/nitrate levels by EGRac status showed differences between CTs.

Comparing plasma nitrites/nitrates via EGRac ratio illustrated a difference in CTs ($p = 0.06$), but not TTs ($p = 0.39$) or CCs ($p = 0.34$). Plasma nitrites/nitrates and MTHFR genotype were not a predictor of SBP ($p=0.4$) but the interaction between MTHFR and plasma nitrites/nitrates was significant in males ($p=0.05$). DBP was not associated with plasma nitrites/nitrates in males ($p=0.3$), but was in females ($p=0.04$). The interaction between MTHFR and nitrates/nitrites was not a predictor of DBP in either sex.

Discussion

This cohort study investigated the relationships among the MTHFR C677T genotype, riboflavin and BP in healthy young adults (age 18-40 years). In this small study, MTHFR genotype was not associated with SBP or DBP, despite literature supporting this relationship [221, 368]. We did see a difference in BP between males and females which aligns with what is recognized in the literature [193-195].

In other cohorts, individuals with a TT genotype demonstrated greater BP than their CC counterparts. In this cohort, we did not find significant differences between SBP and DBP even when controlling for riboflavin status. Riboflavin levels differed between genotypes in males, but not females, in this cohort, and a difference in BP was still not evident. This is contrary to the suggested relationship seen in other MTHFR studies where riboflavin status influenced MTHFR's association with BP [222, 376][328].

To investigate the influence of riboflavin on genotype, we utilized two different measures of riboflavin status: EGRac and plasma riboflavin. Neither was associated with BP in this cohort. To date, EGRac has been used as a gold standard for determining an individual's riboflavin status [377, 378]. It is possible that plasma riboflavin is not as strong an indicator of riboflavin status as measurements in red cells [374, 379]. Plasma riboflavin also did not correlate with EGRac values, though other investigators have found an association between these two measures [374].

Interestingly, EGRac was associated with plasma homocysteine levels within each genotype. Previous work shows that individuals with low plasma folate levels and the TT genotype have elevated fasting levels of homocysteine [380]. While we did not measure plasma folate in this study, the estimated dietary intake of folate was lower in the TT group and plasma homocysteine was higher. In this cohort, plasma homocysteine was associated with SBP when controlling for MTHFR genotype, but it was not associated with DBP. This corresponds with literature finding elevated BP in those with higher levels of plasma homocysteine [212, 218, 292, 381]

We measured plasma nitrate and nitrite as indicators of NO production to determine if there are associations among NO, MTHFR genotype, riboflavin and BP. Plasma nitrates/nitrites were statistically different among the MTHFR genotypes in males, and the interaction between plasma nitrites/nitrates and MTHFR was a significant predictor of SBP in males. Since EGRac ratio was not a predictor of nitrates/nitrites across genotypes, we are not able to suggest a role for riboflavin in the relationship between MTHFR and BP regulation.

Summary

In this study population, we found differences in BP by sex, but not among MTHFR genotypes. Riboflavin status was associated with plasma homocysteine levels, but not BP. Data from this cohort did not support previous findings on the relationship between MTHFR, riboflavin and BP.

CHAPTER 5 OF THE DISSERTATION: Differences in Acute Blood Pressure Response to Sodium Ingestion between MTHFR Genotypes in Healthy Subjects

Introduction

Despite extensive research on hypertension (HTN), increases in blood pressure (BP) continue globally and HTN remains the 10th leading cause of death [175]. Many genes are associated with long term BP increases and HTN [201, 204, 205]. One of these, methylenetetrahydrofolate reductase (MTHFR), has been linked with HTN in genome wide association studies (GWAS) [202, 203]. The gene codes for an enzyme that reduces methylenetetrahydrofolate (CH₂-THF) to methyltetrahydrofolate (CH₃-THF) which serves as a methyl donor to convert homocysteine to methionine [207]. Several studies link individuals homozygous for a single nucleotide polymorphism in the 677th nucleotide (C→T) of the MTHFR gene with cardiovascular disease (CVD) and HTN [382]. This polymorphism produces an enzyme that has a reduced affinity for its substrate, CH₂-THF, and its cofactor, Flavin adenine dinucleotide (FAD), and limits availability of CH₃-THF for conversion of homocysteine to methionine, particularly when folate or riboflavin status is suboptimal [207]. The resulting hyperhomocysteinemia is correlated with damage to the vascular system and linked with diabetes [383, 384], Alzheimer's disease [385], CVD [386], and stroke [387].

This link between MTHFR and CVD and other vascular diseases via homocysteine, however, does not explain the association of MTHFR TT genotype with BP. Recent intervention studies in which B vitamin supplements are used to lower homocysteine levels report no effect on BP [219, 220]. Cohort studies in Northern Ireland have found associations in individuals homozygous for the MTHFR 677TT variant with higher BP across ages from 20 to 60 years old [221]. This association is exacerbated by low plasma riboflavin levels since FAD is a cofactor for MTHFR. Intervention studies in these cohorts have proven successful in lowering BP with

riboflavin supplementation [223, 224]. Therefore, a homocysteine-independent mechanism, possibly related to riboflavin may be responsible for the link between MTHFR and BP.

In these studies, BP has been examined as a static measure or at a few points over time. While this offers a record of long-term change in BP that can be related to an individual's genetic and nutritional characteristics, it does not provide a dynamic picture of how MTHFR may be affecting BP. One of the key factors that influences BP is sodium intake. Chronic high sodium intake is also associated with elevated BP [154, 318], HTN and CVD. Yet the mechanisms underlying these associations are unknown. Despite dietary guidelines proposing reduced sodium diets, a meta-analysis showed that in randomized controlled trials, a reduction in sodium did not reduce the risk of developing elevated BP or HTN [20].

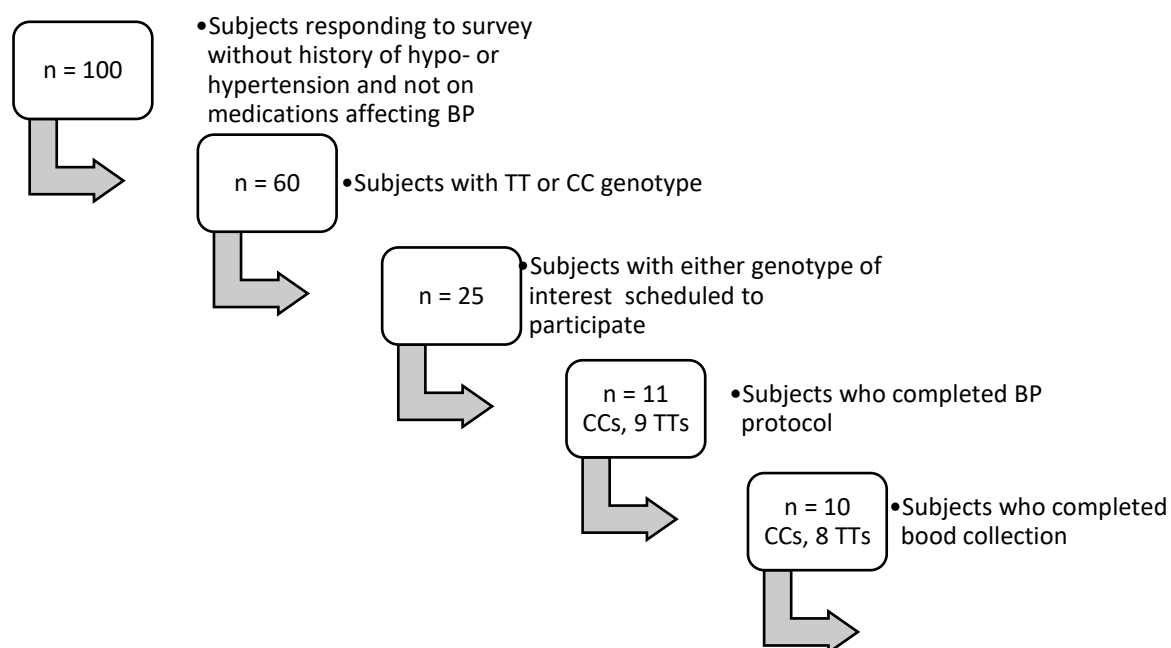
Previous studies from our group showed that acute BP (within 60 minutes) in healthy subjects decreases from baseline following salt or water ingestion and that this decrease is greater following salt ingestion. There is evidence that smooth muscle relaxes, in vitro, when washed with a solution of high sodium concentration [317] and that increased smooth muscle tone over time may play a role in developing hypertension [323]. The methodology previously developed by our group provides insight to the ability of an individual to accommodate for arterial resistance. To determine if this homeostatic mechanism may relate to the observed association between MTHFR genotype and BP, we compared the acute BP responses to sodium ingestion in individuals who are MTHFR CC to those who are MTHFR TT. Our hypothesis was that individuals with TT genotype would have an attenuated acute BP response to sodium intake.

Methods

Subjects: Subjects were recruited from previous studies and via flyer distribution across Rutgers campuses. Subjects were screened to exclude any with a history of hyper- or hypo-tension or taking medications affecting BP. Those qualifying provided a buccal swab for DNA analysis.

Subjects homozygous for the wild type (CC) and variant (TT) alleles of MTHFR 677 were screened via phone or email and were excluded if they were not willing and able to provide a blood sample or if they had a history of hypo- or hyper-tension. Prior to participation subjects read and signed a consent form. Of 100 subjects screened, 60 had genotypes of interest and 25 of these agreed to participate in the study. Of these 20 attended and completed all BP collection appointments, 18 completed all blood collection appointments (Figure 34). The study was approved by the Rutgers University Office of Research Regulatory Affairs, Institutional Review Board.

Figure 34: MTHFR Acute BP Study Recruitment and Retention Data



Acute BP Protocol: Subjects arrived fasted for at least 8 hours for an appointment at 8am or 10am. BP was measured via manual sphygmomanometer and stethoscope reading on left arm after a seated rest period of 10 minutes. Two resting BP readings were taken 10 minutes apart (-20 and -10 min) and a bolus of sodium chloride in water or water was given at time 0. BP was taken for 60 minutes following the bolus ingestion at 10 min intervals. Subjects recorded mood

physiology and cravings at each BP measurement (see form in Appendix A4). This protocol was repeated 3 times for each bolus per subject on different days.

Stimuli: Solutions were prepared within 1 week of use using NaCl (99%, Sigma S5886) and Millipore deionized water. The water solution was 475 mL of deionized water and the salt solution was 475 mL of 157 mM NaCl (1.9 g sodium) in deionized water. All solutions were warmed to 30°C prior to subject ingestion.

Questionnaire: Upon beginning the study, subjects completed a diet and lifestyle questionnaire to ascertain their typical sodium intake and factors that may affect their BP (see form in Appendix A6). Subjects provided liking, quantity and frequency of intake for foods high in salt such as cheese, table salt, and canned soups and riboflavin/folate including dairy products, leafy greens and legumes. The questionnaire also asked basic health information including frequency of exercise, drug use and previous BP diagnosis of hypo- or hyper-tension.

Buccal Swab DNA Analysis: Buccal swabs were frozen until analysis. Upon thawing, tissue was collected and DNA analyzed using Life Technology's Taqman© sample to SNP kit on RT-PCR.

Blood collection and processing: On days separate from BP measurement, subjects arrived fasted for at least 8 hours for an appointment at 8am or 10am (the same appointment time as their BP measurement was done). A certified phlebotomist drew 9 mL of blood into an EDTA tube and 6 mL into a serum separator tube at each of two baseline draws (-20 and -10 minutes). Blood collection was completed 1 time per stimulus, per subject, on different days. Following collection, serum remained at room temperature for 30 minutes until centrifugation; the serum layer was removed into aliquots for freezing. EDTA plasma tubes were held on ice until centrifugation, followed by removal of plasma and buffy coat and washing of red blood cells. All samples were stored at -80°C until analysis.

Blood Analysis: Riboflavin levels in the plasma were quantified via HPLC based on previously published methods [370, 388]. Homocysteine was quantified via HPLC following methods previously published [389, 390]. Plasma sodium levels were measured using a Coleman flame photometer following the manufacturer's protocol.

Statistics: Using Stata and excel, data were analyzed for associations between average SBP, Δ BP and MTHFR 677 genotype using ANOVA. Comparisons between groups and conditions were tested using two-way ANOVA. Influence of subject characteristics was calculated using multiple regression analyses. Significance was considered $p < 0.05$.

Results

Subject Characteristics: A total of 11 CCs and 9 TTs, age 18 to 40 years, participated in the BP protocol. All subjects were healthy and ambulatory, having no history of hyper- or hypo-tension or any medications affecting BP. The mean age of all subjects combined (irrespective of MTHFR genotype) was 23.1 ± 6.1 years and mean BMI was 24.6 ± 6.0 . Mean age and BMI did not differ between MTHFR genotype groups ($p=0.2$ and $p=0.3$, respectively). A similar ratio of African, Asian, Caucasian and Hispanic participants was included in each group and each group contained about an equal ratio of male and female participants (Table 7).

Resting BP was calculated as an average of the resting BP readings taken on all trials for each subject. This average was then averaged within each genotype group. There were no significant differences between resting systolic or diastolic pressure between the two groups (Table 7). The overall cohort average resting systolic BP was 106.5 ± 11.6 mmHg and average resting diastolic was 66.35 ± 8.93 mmHg. Average resting SBP in CCs was 107.2 ± 13.7 mmHg and in TTs was 105.7 ± 9.1 mmHg. Average resting DBP in CCs was 67.7 ± 10.9 mmHg and in TTs was 64.7 ± 6.0 mmHg.

Plasma homocysteine and riboflavin were measured in baseline blood samples. Neither plasma homocysteine or plasma riboflavin were statistically different between genotype groups (Table 7).

		TOTAL	CC	TT	<i>p</i>
n		20	11	9	
BMI		24.6 ± 6.0	26.0 ± 5.6	23.0 ± 5.9	0.3
Age (years)		23.1 ± 6.1	25.0 ± 7.3	21.0 ± 3.6	0.2
Sex	M	11	6	5	
	F	9	5	4	
Ethnicity	African	4	2	2	
	Asian	5	2	3	
	Caucasian	9	6	3	
	Hispanic	2	1	1	
Resting SBP (mmHg)		106.5 ± 11.6	107.2 ± 13.7	105.7 ± 9.1	0.8
Resting DBP (mmHg)		66.35 ± 8.9	67.65 ± 10.9	64.76 ± 6.0	0.5
Plasma Riboflavin (nmol/L)		16.92 ± 9.7	16.4 ± 6.0	15.4 ± 11.6	0.7
Plasma Homocysteine (umol/L)		11.9 ± 5.5	11.8 ± 4.3	11.5 ± 4.3	0.8

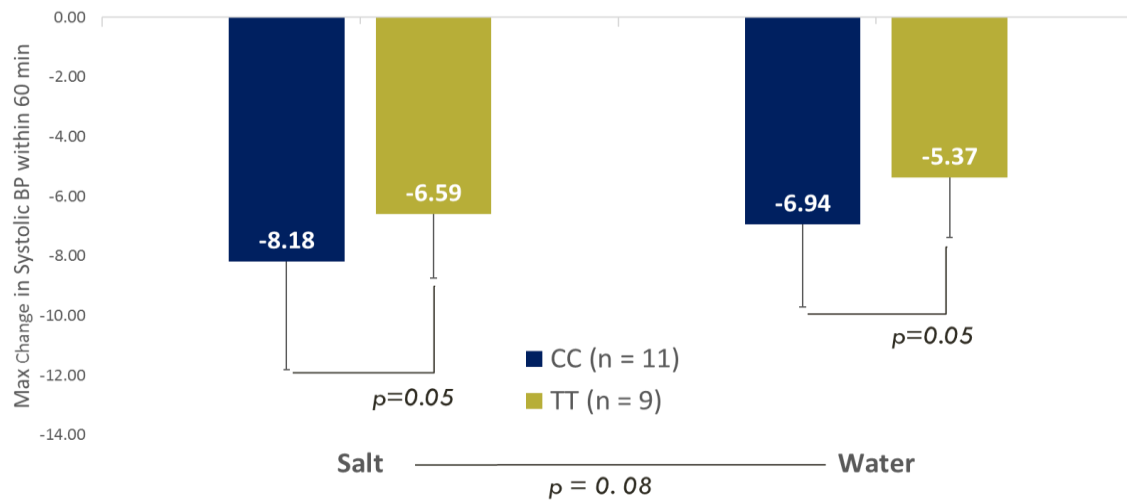
Table 7: MTHFR Acute BP Study Subject Characteristics

Average BP post ingestion: We averaged BP post ingestion for comparison with resting BP. To calculate post ingestion BP, we averaged all BP data points, following bolus ingestion, in a trial for a subject and then averaged these values for all three trials per stimuli. Taking an average of the whole cohort, we observed a similar SBP following a salt (104.5 ± 11.7 mmHg) or water (104.8 ± 11.8 mmHg) rinse compared to resting SBP (106.5 ± 11.6 mmHg) ($p=0.9$). We observed no significant differences in DBP post salt (68.7 ± 9.9 mmHg) or water (68.7 ± 9.3 mmHg)

compared to resting DBP (66.35 ± 8.9 mmHg) ($p=0.2$). Between groups, there were also no differences in average SBP ($p=0.6$) or DBP ($p=0.8$) post ingestion.

Acute change in BP: Average BP post rinse does not account for transient decreases and return to baseline that occurs within 60 minutes of salt or water ingestion. So, to measure the group acute BP response, we calculated the average max decrease in BP for each individual and averaged these within the genotype groups. Mean SBP decreased (irrespective of MTHFR genotype) after consumption of the salt (-7.5 ± 3.1 mmHg) or water bolus (-6.2 ± 2.6 mmHg) ($p=0.08$). The reduction in SBP in response to both sodium and water ingestion in the TTs was less than in the CCs ($p=0.05$) (Figure 35).

Figure 35: MTHFR Acute BP Study Change in SBP Post Salt Ingestion by Genotype

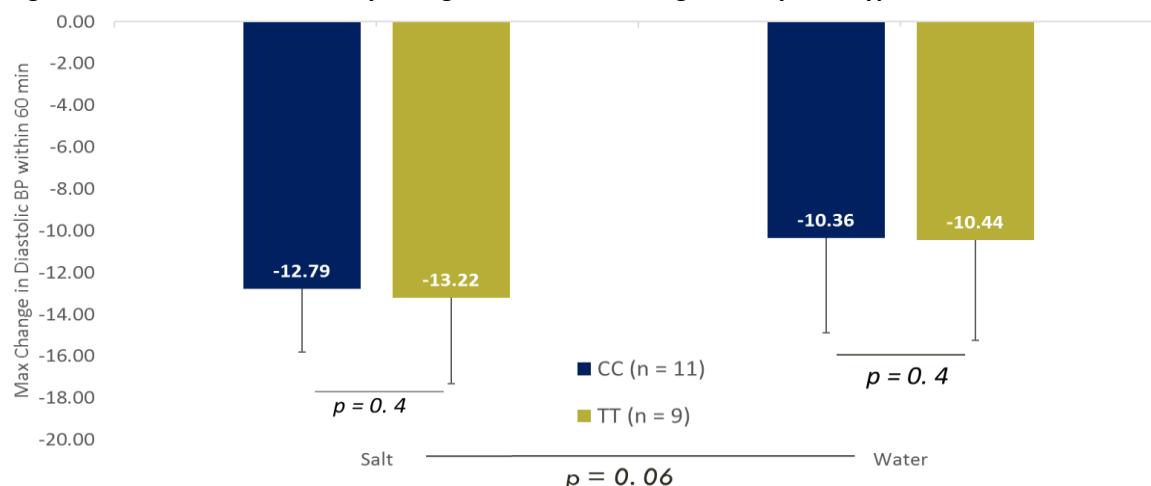


Change in SBP was lower following ingestion of salt (left) compared to water (right). TTs (gold bars) responded less compared with CCs (blue bars).

In contrast, no differences between MTHFR genotypes were observed in DBP in the response to salt or water ingestion ($p = 0.4$) (Figure 36). Though there were not differences

between genotypes in DBP response, mean DBP response of all subjects after salt ingestion (-12.3 ± 4.3 mmHg) was greater than after water ingestion (-10.1 ± 4.7 mmHg) ($p=0.06$).

Figure 36: MTHFR Acute BP Study Change in DBP Post Salt Ingestion by Genotype



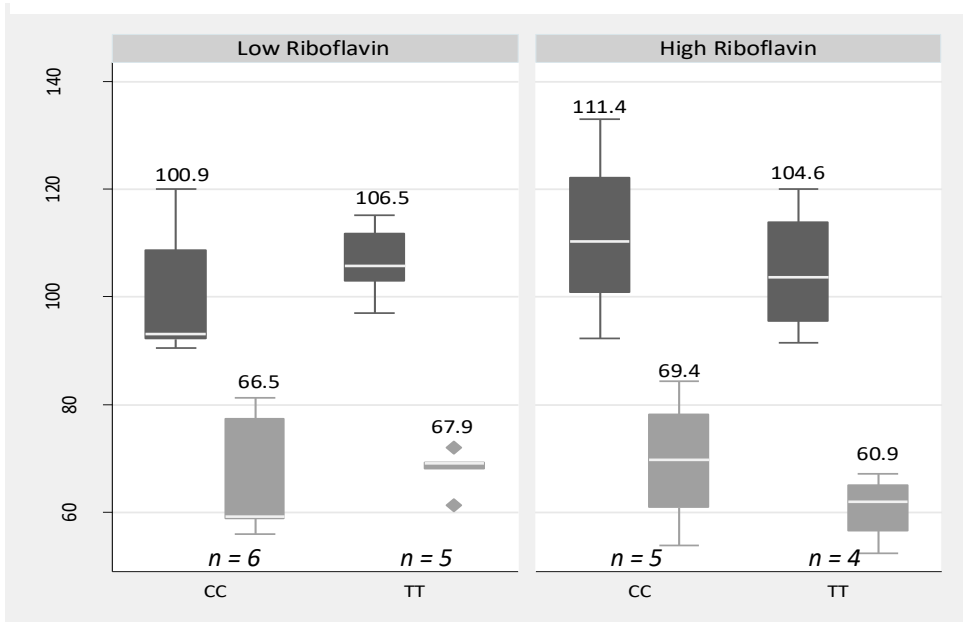
Change in DBP was lower following ingestion of salt (left) compared to water (right). Response was similar between TTs (gold bars) and CCs (blue bars).

Influence of plasma riboflavin on BP: Riboflavin has been shown to influence the relationship

between

Figure 37: MTHFR Acute BP Study Resting BP by Genotype and Riboflavin

MTHFR and
BP. While
we did not
find
significant
differences
between
group
averages of
riboflavin



Riboflavin influenced SBP (dark boxes) and DBP (light boxes) within MTHFR 677 groups.

Though not significantly different ($p = 0.4$), but the pattern exists in DBP and SBP.

(Table 7), there was a range of values in this cohort, from 4.63 – 41.7 nmol/L. To see if there was an influence from riboflavin on BP in this cohort, we divided each genotype into high (above the group average) or low (below the group average) riboflavin. When we plotted resting SBP and DBP, we observed differences in BP within the low and high riboflavin groups, but did not find significant associations between genotypes ($p=0.4$) (Figure 37).

However, when we used ANOVA to compared resting BP with MTHFR and plasma riboflavin values (not a high/low categorization) we found significant associations. We found similar associations with average SBP and DBP post ingestion, but not with the change in SBP or DBP post ingestion (Table 8).

		Model R²	MTHFR	Plasma riboflavin	MTHFR x plasma riboflavin
Average Resting SBP		0.6	0.002	0.02	0.0004
Average Resting DBP		0.6	0.01	0.03	0.0009
Average SBP Post Ingestion	Salt	0.7	0.001	0.006	0.0002
	Water	0.6	0.003	0.02	0.0005
Average DBP Post Ingestion	Salt	0.5	0.02	0.13	0.003
	Water	0.5	0.02	0.12	0.003
Average Change in SBP Post Ingestion	Salt	0.04	0.5	0.4	0.9
	Water	0.05	0.7	0.5	0.2
Average Change in DBP Post Ingestion	Salt	0.07	0.6	0.6	0.5
	Water	0.02	0.9	0.4	0.6

Table 8: MTHFR Acute BP Study ANOVA Results

Discussion

The MTHFR 677TT genotype has been associated with elevated BP, and we sought to determine if this variant genotype was associated with a diminished BP response post salt ingestion. Acute blood pressure responses to salt ingestion are influenced by MTHFR 677 genotype. Individuals with the TT genotype displayed a reduced decrease in SBP compared with their CC counterparts. No difference between genotypes was observed for the decrease in DBP after salt ingestion. SBP is considered the primary indicator of risk for hypertension, though DBP is also important for risk determination [391]. Systolic pressure changes are directly related to arterial flexibility and are influenced by nitric oxide (NO) production [392]. The attenuated SBP change in TTs in response to salt ingestion suggests an impairment of a reflexive pathway that is influenced by MTHFR. A link between MTHFR and NO has been shown in patients with TT genotype that had less NO bioavailability during bypass graft surgery [393].

Resting SBP and DBP were slightly lower in TTs, though not at a statistically significant level. This may be a result of the small number of subjects in this study since other work has shown a lower DBP in CCs compared with TTs [217]. Lower DBP in times of increased arterial resistance may be an indicator of reduced arterial stiffness [394].

MTHFR's effect on BP has previously been seen more strongly in the presence of low riboflavin levels [221, 224]. In this cohort, riboflavin levels were comparable to published levels in healthy individuals [374], and not significantly different between CC and TT genotype groups. However, when plasma riboflavin and MTHFR genotype were combined, they accounted for a significant portion of the variance in average SBP and DBP post salt ingestion. It is possible that sodium is providing a stressing effect on the mechanisms involved in maintaining BP similarly to low levels of riboflavin in these other studies. The knowledge that both nutrients relate to the MTHFR effect on BP may aid future work in pinpointing the full mechanism.

To address the initial hypothesis that MTHFR is linked with BP via homocysteine, we measured homocysteine in plasma at baseline and found no between CCs and TTs. We also found no association between BP and homocysteine levels in these subjects. While in other cohorts of hypertensive and older individuals, homocysteine was associated with BP [269, 395], in these young, healthy individuals we did not see evidence for a link between homocysteine and BP. Studies investigating the relationship between homocysteine and BP in smaller, healthy cohorts have also found a similar non-association [396, 397].

Summary

This is the first study to investigate the effect of a BP related gene on acute BP response. Our results provide evidence that MTHFR 677 genotype impacts the acute BP response to sodium ingestion. The impaired SBP response displayed by the TTs suggests an effect of MTHFR 677 on the immediate regulation of BP. The findings presented here indicate a mechanism for MTHFR's effect on BP via regulation of vascular tone. Though we did not directly measure the pathways that directly regulate BP, in the future, this insight would be useful in pinpoint the pathways that lead to MTHFR's effect on BP changes.

CHAPTER 6 OF THE DISSERTATION: Conclusions and Recommendations

Through this document, new findings regarding BP, sodium and MTHFR have been presented. The relationships and implications between these data and what they add to current literature are reviewed here. This chapter summarizes the work presented and provide its limitations, synthesize their implications, and suggest future research directions.

Summary of Findings

Politicians, health professionals and scientists around the world hope to improve the health and global quality of life. Since elevated BP is one of the leading causes of death and has a strong relationship with other lifestyle diseases, it is the subject of much policy and research work. Despite the body of literature seeking a cause for essential HTN and for the link with salt intake, conclusions are slim and many questions are unanswered. **Study of BP under dynamic conditions, rather than at rest, can provide insight to the effectiveness of an individual's BP regulation.** The goal of these studies was to understand the acute BP effects of sodium ingestion, to determine if there is an anticipatory effect and to understand the impact of MTHFR genotype on static and acute BP response. These studies provide a greater understanding of how BP homeostasis relates to MTHFR, riboflavin status and salt intake.

In multiple studies, an acute BP response was demonstrated. A greater decrease in BP followed salt ingestion compared with water ingestion. In two different cohorts, this response was shown as a repeated, reliable trait. Additionally, BP decreased following the taste of salt alone, suggesting an anticipatory BP effect. These acute BP changes occur within the first 60 minutes of sodium ingestion or taste and appear to function as homeostatic maintenance.

How MTHFR influences BP and under what conditions is a question that this work sought to help answer. In a cohort of healthy subjects with normal dietary riboflavin and folate intake,

MTHFR was not shown to influence static BP. However, in a similar cohort of healthy subjects, MTHFR did influence acute BP response; individuals with the TT variant demonstrated a lesser decrease in BP compared with individual expressing the wild-type CC genotype.

Together, these data answered the questions posed by illustrating an acute BP response, and showing the influence of MTHFR on both static and acute BP. There are a few significant findings beyond each study's individual conclusions that came from this thesis. First, the finding that BP demonstrates a measurable decrease in BP following water and sodium ingestion. Second that study of acute BP highlights differences in BP regulation that resting BP measurement did not. Third, that plasma riboflavin plays a role with MTHFR in influencing acute BP, even in individuals with sufficient riboflavin intake.

The decrease in BP illustrated in two studies, was a surprising finding given the known association between salt intake and elevated BP. In these studies, the blood volume and renal changes were not studied; however, Guyton's model of renal regulation predicts that changes in sodium levels in the extracellular fluid will cause shifts in BP [64, 132]. In this model, a shift to elevated BP (over time) is suggested. However, this assumes a precise system, where exactly the right amount of blood volume and pressure are adjusted to account for a sodium load. The patterns seen in the acute BP studies imply that most individuals operate with imprecision and therefore, BP fluctuates to maintain homeostasis: average BP is maintained via some initial peaks and valleys.

Because the amount that BP decreases acutely could be an indicator of how well an individual regulates BP and sodium balance it may be used as an indicator of risk status. Resting BP measurement in the original MTHFR cohort was not associated with risk, despite this association in other studies [221, 223, 224]. However, the decrease in acute BP following salt

ingestion was less in individuals with the variant TT genotype, a known risk factor for elevated BP. The utilization of the acute BP measurement illustrated an association between BP and an at-risk genotype in healthy individuals. A blunted BP response to salt ingestion may indicate a limited ability to regulate and overtime this altered regulation could shift healthy BP to HTN.

In both MTHFR cohorts, folate and riboflavin dietary intake was above adequate intake. Riboflavin plasma levels were normal and not associated with MTHFR genotype. However, an interaction with riboflavin and MTHFR 677 genotype was highlighted with the acute BP response. FAD, a derivative of riboflavin, is a cofactor for MTHFR and NOS, which also requires FMN, another riboflavin derivative. The availability of riboflavin in the plasma influences the effectiveness of both enzymes, and may influence vasodilation of blood vessels.

Impact of Findings on Current Literature

Current understanding of BP and its relationship to sodium intake is limited, despite a large body of literature on the subject. The data presented in this work, suggests a unique methodology that can indicate risk for dysfunctions in acute BP regulation (NOS and RAAS). The study of acute BP integrates knowledge of pulmonary-renal regulation [398] with the clinical impact of salt intake. Combining the knowledge from these two fields provides a more complete picture of how essential HTN develops. Resting BP measurement, one measurement per day/week/month/year, provides an incomplete picture of a dynamic system. This thesis showed that two groups of subjects may appear healthy given a resting BP measurement, but had different abilities to regulate BP under a high salt condition.

MTHFR is a gene of interest related to BP through epidemiological and genome-wide association studies. This thesis suggests that BP regulation is affected by MTHFR's enzyme product. TT's in this study demonstrated a diminished decrease in acute BP compared with CC's. The relationship between MTHFR and acute BP was also influenced by riboflavin, supporting the

current body of literature on this topic. A few other studies suggest a relationship between the MTHFR TT variant and reduced NO production [399, 400]. Riboflavin's products, FAD and FMN, are important cofactors for NOS. Validation of the MTHFR-riboflavin interaction suggests that MTHFR's influence on BP may be via NOS.

Limitations of Dissertation Work

The work included in these chapters provides insight into the science of BP measurement, sodium chloride intake and one carbon metabolism. While there were statistically significant findings, the design of the studies limited the conclusions possible. The cohorts in all studies were small, making these only pilot findings. The size of each study provided differences between genotypes, but was limited in providing statistical information about sex, age and ethnic groups.

In these studies, subjects' responses were tested over the course of a few months, rather than at one appointment. Our findings validated the reliability of the acute BP response. However, we cannot offer conclusions about how this acute response leads to development of HTN. Additionally, we cannot make conclusions about the way these responses may change in older adults or those who have other conditions that affect BP.

MTHFR is a gene associated with BP which encodes an enzyme that metabolizes folate. In these studies, information about individual's folate status was collected by dietary recall, but not with red cell folate. Diet recall confirmed that all subjects were eating at least the recommended daily intake of folate, but red cell folate levels might identify individuals undernourished in folate or associations with folate and BP levels.

Applications and Recommendations

Despite the limitations of this research, the conclusions from these studies help to understand the relationships between diet, genes and BP regulation. Further studies based on

these conclusions may uncover the answers needed to prevent essential hypertension and reduce the risk of related deaths.

Because these studies were completed only at the scale of a pilot, a larger cross sectional study would allow the study of acute BP patterns by sex, ethnicity and health state. This study will allow for a greater model of population BP changes in response to sodium chloride ingestion. Additionally, studying these patterns in a cohort across multiple years will validate the reliability of this pattern in an individual and provide an understanding of how these responses change with age.

These studies did not uncover the mechanism linking sodium intake and essential HTN or MTHFR and BP, but did offer clues for future research in this area. Further study of BP following sodium ingestion to measure RAAS, blood volume and NO production would complete the picture of how different individuals manage salt intake.

Further understanding is also needed of the relationships between MTHFR and BP. The data collected suggests there may be an influence of MTHFR and riboflavin on NO levels. Detailed work to determine how these genes, vitamins and pathways relate may offer preventative options for a significant portion of the global population.

APPENDICES OF THE DISSERTATION

Discerning the Effect of Salt, Riboflavin and MTHFR on Acute Blood Pressure Response

by MELISSA ANNE MURPHY

Dissertation Directors:

Joshua W. Miller and Paul A.S. Breslin

A1: Chemical Structures of Key Molecules

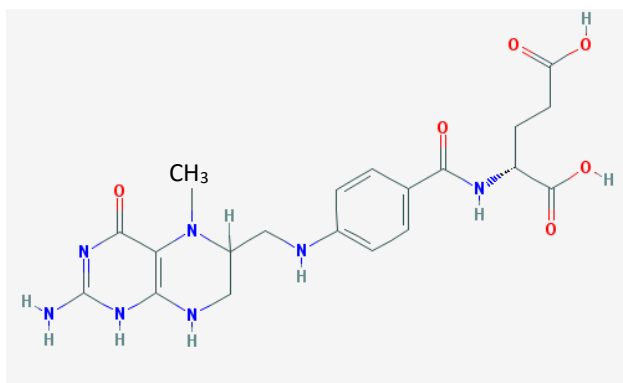
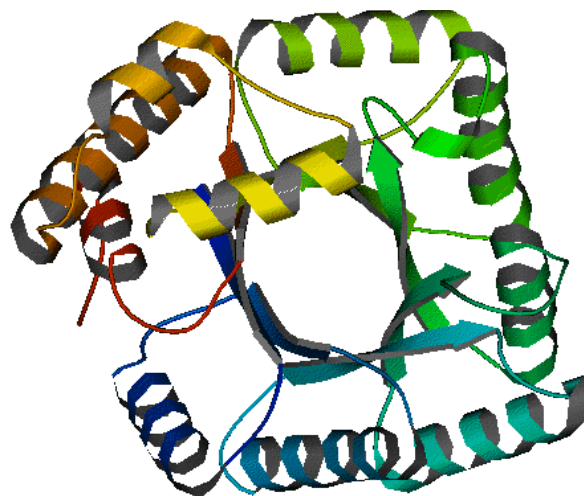
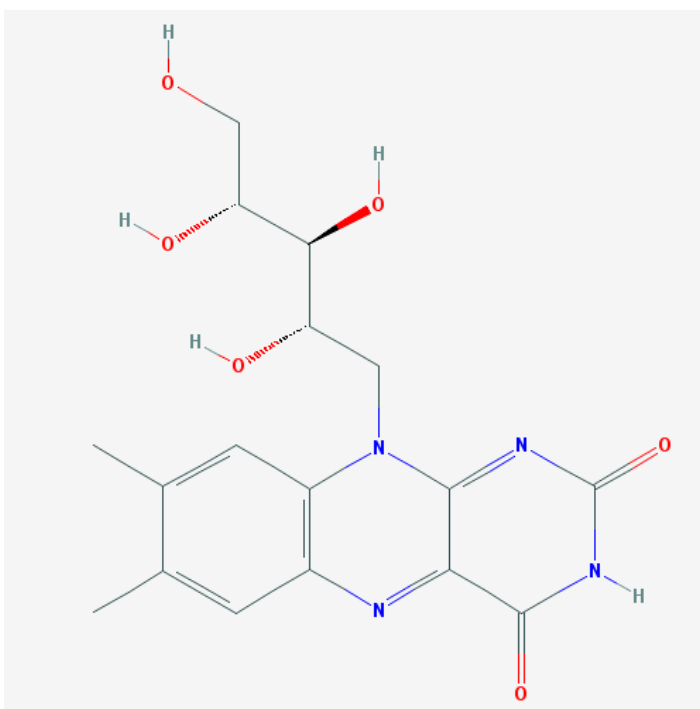
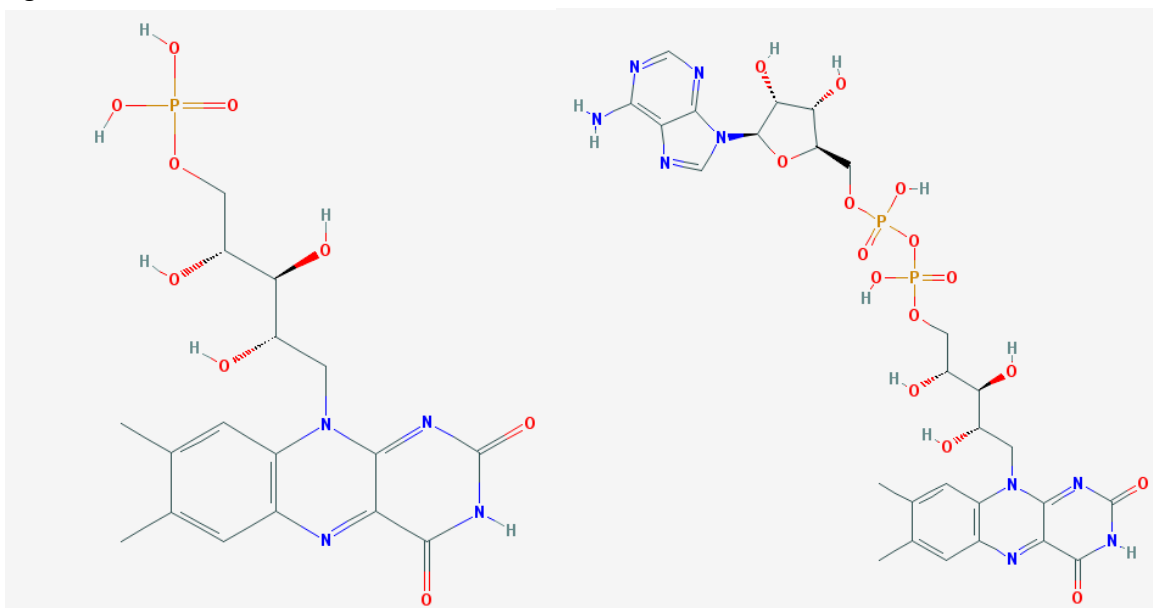
Figure 38: Chemical Structure of 5-MTHF*5-methyltetrahydrofolate is the end product of MTHFR [401]***Figure 39: Chemical Structure of MTHFR***5',10'-Methylenetetrahydrofolate Reductase [402]*

Figure 40: Chemical Structure of Riboflavin

Riboflavin is a water soluble vitamin [403]

Figure 41: Chemical Structure of FMN and FAD

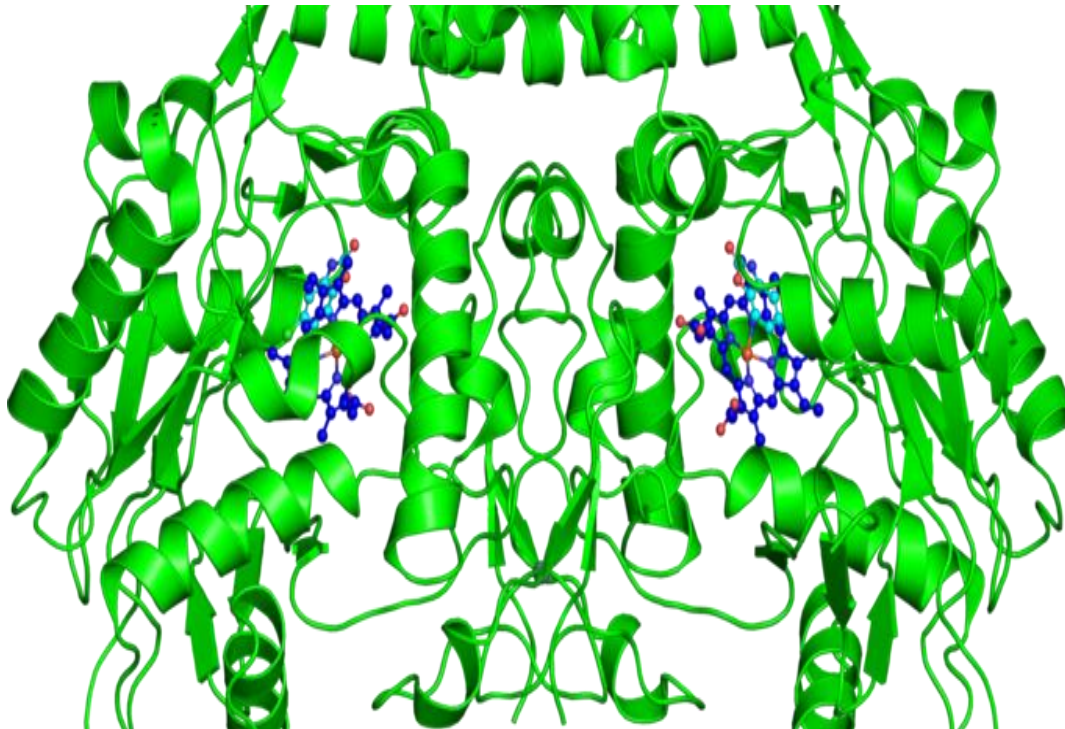
Flavin Mononucleotide (left) [404] and Flavin Adenine Dinucleotide (right) [405] are synthesized from riboflavin

Figure 42: Chemical Structure of Nitric Oxide



Nitric Oxide is the end produce of Nitric Oxide Synthase action on L-Arginine [406]

Figure 43: Chemical Structure of Nitric Oxide Synthase



Human Endothelial Nitric Oxide Synthase Structure: Enzyme and cofactors colored separately [407]

A2: Detailed Recruitment and Laboratory Methods

Recruitment of subjects for acute sodium ingestion: Study personnel posted IRB approved fliers throughout Rutgers campus facilities, in applicable posting locations. Interested participants contacted study phone and email for screening. Study personnel contacted potential subjects to determine their eligibility. If participants qualified, they were scheduled for and six 2-hour sessions, maximum two per week with at least 1 day in between testing days. Personnel verbally and electronically shared the following information with participants:

“Please fast after midnight, no food or drink besides plain water. You may eat and drink freely at the conclusion of the study period at 8:30am. You may bring something with you to read or a laptop to use, provided these materials will not affect your mood or stress level.”

Inclusion/Exclusion criteria: Subjects were excluded if they smoked, were on medication for blood pressure treatment or if they were unable to give blood. All other healthy subjects who were able and willing to provide blood were scheduled for appointments.

Study procedures: On the first day, subjects arrived in at the testing room and were asked to sit in an upright position. Study personnel gave subjects an IRB approved consent form and allowed time for reading and questions. Once the consent form was signed, personnel described the study to the subject:

“You will be asked to sit in an upright, relaxed position for 2 hours. During this time, a blood pressure cuff will be placed and will remain on your left arm. Every 10 minutes, your blood pressure will be taken. You will be given a drink an asked to finish it between blood pressure readings. You are also asked to record any changes from one reading to the next in mood, physiology and

cravings; mood would indicate “happy” or “sad”, etc. Physiology ‘tired,’ ‘anxious,’ ‘hungry,’ etc. Cravings are any specific food or flavor you may be craving. You may read or utilize an electronic device provided that it does not affect your mood or stress level. Do you have any questions?”

MTHFR Subject Screening: Subjects emails and phone calls were returned with the following script by study personnel.

“Thank you for your interest in this study. The purpose is to evaluate the relationship between blood pressure, diet and related genes. If you decide to volunteer, you will be asked to fast after midnight the night before your appointment. When you arrive, you will first complete a brief demographic and diet questionnaire. Then we will record your height and weight and blood pressure. We will finally obtain a 15-mL blood sample from a vein in your arm. The amount of time asked is approximately 30 minutes during one visit. You will be paid \$15 for completion of these steps.

I am going to ask you a series of questions to ensure that you qualify for the study:

What is your name?

What is a current email address where we can reach you?

Are you 18 or older? (must be “Yes”)

Do you weigh at least 110 lbs? (must be “Yes”)

Are you pregnant or trying to get pregnant? (must be “No”)

Are you willing and able to provide a blood sample? (must be “Yes”)

Can you commit to 30 minutes of time and participate in the described study?

(must be “Yes”)

If all appropriate answers provided above, find a date and time that the subject is available; write it on this sheet and schedule it on the study calendar. Tell the subject the following:

You are confirmed to participate in this study on _____ at _____ . Please arrive a few minutes early to Room 324A of the Food Science and Nutritional Sciences Building on Cook Campus at Rutgers University. You will receive a confirmation email at the address you provided. Thank you and see you then!”

MTHFR Sodium Bolus Screening: “Thanks for your interest in this study! The purpose is to evaluate the relationship between blood pressure, diet and related genes. If you qualify and decide to volunteer, you will be asked to participate in eight 90 minute sessions, taking place in the Food Science Building on Cook Campus. At your first session, you will be asked a series of brief demographic, medical and diet questions. During the first 6 sessions, you will fast after midnight the night before and will be given a drink of water or sodium chloride in water while your blood pressure is taken during the 90 minutes. For the remaining two sessions you will fast after midnight before and will be given a drink of water or sodium chloride in water while your blood is drawn periodically during the 90 minutes, and you will be asked to collect urine for 24 hours following these two sessions. In total, your participation should last 4 weeks, with two sessions per week. You will be paid \$15 per session for blood pressure measurement, \$25 per session for blood draw procedures. You will receive a \$10 bonus for completing all eight sessions. If you withdraw before completion of the studies, you will receive payment of all sessions completed to that point. Can you please answer the following questions so I can ensure that you qualify?”

1: Are you at least 18 years of Age? [Yes]

2: Do you weigh at least 110 lbs? [Yes]

3: Are you pregnant or trying to get pregnant? [No]

4: Are you willing and able to provide blood and urine samples? [Yes]

5: Are you taking any medications for the treatment of hypertension, hypotension or other blood pressure related conditions? [No]

6: Do you have hypertension, hypotension or other blood pressure related conditions?
[No]

If subject meets all requirements above, he/she will be asked to come by lab to provide cheek swab for genotype screening. If genotype is one of interest, see below.

Based on the bracketed desired response in the right column, determine if the person qualifies.

If no, "Thank you for your interest; however, you do not qualify to participate in this study."

If yes, "You qualify for the study and we are able to schedule you for your first appointment on __ (day) __ or __ (day) __ at __ (time) __. Please fast after midnight the night before your appointment (no food or drink except for water) and arrive a few minutes prior to your scheduled time to review a consent form and study procedures.

The study will take place in the Food Science Building, Room 305, on Cook Campus. We will send you these instructions and confirmation via email." Obtain an email address and phone number for confirmation.

Time Course Blood Pressure Readings: Once subjects signed consent forms and acknowledged understanding of the study procedures, study personnel placed a blood pressure cuff on the left arm, immediately above the elbow. While holding the arm extended, a manual sphygmomanometer was inflated to 20 mmHg past the point when no sound is heard through a stethoscope. Study personnel recorded Systolic pressure as the first pressure at which an audible sound is heard as the cuff is deflated. Diastolic pressure is recorded as the first pressure at which no further sound is heard through the stethoscope as the cuff continues to deflate. Study personnel recorded blood pressure 20 and 10 minutes prior to bolus ingestion, at the time of bolus ingestion and 10, 20, 30, 40, 50 and 60 minutes following bolus ingestion.

Sodium Ingestion Bolus: In a randomized order, study personnel gave subjects either 475 mL of deionized water or 475 milliliters of 157 millimolar sodium chloride solution. Over the course of six sessions, subjects received each solution three times.

Salt Diet Questionnaire: During each session, subjects utilized the form in Appendix ____ to record any changes in their physiology, mood and cravings at each blood pressure recording. If no change occurred, subjects noted a line. Additionally, during the first session, subjects completed the salt diet questionnaire (Appendix ____) to describe typical health and eating behaviors.

Controls for Sodium Ingestion Study: The same stethoscope and sphygmomanometer were used for a subject from session to session to reduce instrument variability between sessions. Subjects serve as their own control for each condition.

MTHFR Subject Appointment: Each participant will attend one 30 min session where all data will be collected. Following consent, the session includes the following components:

MTHFR Blood Pressure Measurement: Subjects arrive having fasted, no food or drink except water, after midnight the night prior, and rest in an upright seated position for five minutes prior to a mercury sphygmomanometer being placed on their upper left arm just above the elbow. Blood pressure is measured 3 times waiting briefly in between, in accordance with NHANES procedures [43].

Riboflavin and Folate Diet Questionnaire: Each subject will complete an IRB approved health and diet intake questionnaire. Questions include known medical conditions, exercise frequency and frequency of Riboflavin and Folate rich foods.

Anthropometrics Measurement: Subjects height is measured using a wall marker and weight is measured with an electronic scale.

Blood Collection: Blood is collected by a certified phlebotomist using a 21G butterfly needle. One 5 mL serum separator tube is collected followed by one 10 mL EDTA plasma tube is collected.

Blood processing: The EDTA tubes were inverted 8 to 10 times to ensure mixing of EDTA with blood and immediately placed on ice for a maximum of 60 minutes. EDTA tube was then placed in a centrifuge for 10 minutes at 1300 rpm. The top plasma layer was removed into 0.5 mL aliquots in cryovials which were kept on ice until frozen at minus 80 within 60 minutes. The buffy coat, white layer of cells, was removed and placed in a separate tube also kept on ice until frozen at minus 80 C within 60 minutes.

Physiological Saline (0.9%) was added to blood in equal volume and mixed by inversion. Tube was then centrifuged for another 10 minutes at 1300 rpm. Top layer of fluid was removed into a biohazard waste container. This washing process took place three times; after the third wash, red blood cells at the base of the tube were placed into 0.5

mL aliquots and kept on ice for up to 60 minutes before being frozen at minus 80C. To one aliquot of 100 uL of blood, 900uL of 10% Ascorbic acid was added to stabilize folate for later assay.

Serum separator tubes (SST) sat at room temperature for 30 minutes before being centrifuged for 10 minutes at 1000 rpm. The top serum layer was removed into 0.5 mL aliquots and kept on ice for a maximum of 60 minutes before being frozen at minus 80C.

Hematocrit: Using freshly drawn whole blood from an EDTA tube, a microcapillary tube was filled to designated volume and sealed. The microcapillary tube was placed in a Beckman Counter Statspin centrifuge and spun for 5 min. Tube is then read with the ruler on the microcapillary tube holder.

Erythrocyte Glutathione Reductase Activity Coefficient: All methods for this assay are performed in dim lighting to protect samples from degradation. One aliquot of frozen washed red blood cells was removed from the minus 80 and allowed to thaw in room temperature water for 5 minutes. RBC were then vortexed and 100 uL was removed and placed in an amber Eppendorf tub with 200 uL of DI water. Tubes were vortexed and allowed to sit at room temperature for 10 minutes to lyse cells. Tubes were then centrifuged for 10 minutes at 2000 rpm. At completion, 20 uL of supernatant is added to 1.98 mL of phosphate buffer in a plastic vial, vortexed and kept on ice.

Into two amber Eppendorf tubes per sample, study personnel place 800 uL of diluted sample. Into each tube, 80 uL of phosphate buffer or FAD is added. Samples are vortexed and incubated at 37C for 15 minutes. Keeping samples in incubator, tube is vortexed and 80uL is removed into two wells each (duplicate) on a 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC + FAD		S1 + FAD		S2 + FAD		S3 + FAD		S4 + FAD		Bl. + FAD	
B	QC + Buffer		S1 + Buffer		S2 + Buffer		S3 + Buffer		S4 + Buffer		Bl. + Buffer	

The final two steps are the addition of reaction product and activation co-factor. First 135uL of Glutathione is added to each well. Then 70 uL of NADPH is added to each well and all wells are pipet mixed. Plate is placed in spectrophotometer and read at 340 nm, every 10 seconds for 20 minutes.

Homocysteine: Levels of homocysteine and cysteine in the plasma were measured via HPLC. Samples were thawed and incubated for 30 minutes at room temperature with 1:1 volume of TCEP and 0.5:1 volume of Internal Standard. A 1:1 TCA to sample volume was then added and solution was vortexed and centrifuged at 14000 rpm for 10 min. A working SBDF solution (1mg/ml SBDF in 2mol/L boric acid, 4 mmol/L EDTA) was added to an autosampler vial with the sample volume of sample supernatant and twice the volume of sample prep buffer (2 mol/L Boric Acid, 4mmol/L EDTA, pH 10.0). The solution was incubated for 60 minutes at 60C and then cooled at 4C for 30 min. Samples were then run for 15 minutes each on an Agilent 1200 HPLC with a flow rate of 0.25 mL/min on a C18 Rev Phase column with an excitation wavelength of 385 nm and an emission wavelength of 515 nm.

Calculations are based on a ratio of a known external standard (20 nmol Homocystine and 100 nmol Cystine) peak height compared to a known concentration internal standard (Cystamine) peak height. The equation is:

$$x [Hcy] = ES[Hcy] * \frac{x \text{ Hcy Peak} / IS \text{ Peak}}{ES \text{ Hcy Peak} / ES \text{ IS Peak}}$$

Riboflavin via HPLC: All assay methods take place in dim lighting to protect sample from degradation. An aliquot of frozen plasma is allowed to thaw in room temperature water for 5 minutes. Tube is vortexed and 150 μ L is removed and mixed with 300 μ L of 4C Methanol in an Eppendorf tube. After vortexing, sample is centrifuged at 12,000 rpm for 10 minutes. A 150 μ L aliquot of supernatant is removed into an HPLC autosampler vial and remaining supernatant can be frozen or held at 4C for up to 24 hours. Samples are injected as 50 μ L into a 2.9 pH mobile phase comprised of 13% Acetonitrile and 50 mM NaH_2PO_4 and DI water; the Agilent 1200 HPLC is programmed for excitation 470 nm and emission 530 nm with a flow rate of 0.5 mL/min for 10 minutes and the autosampler temperature held at 15C.

Calculations are based on concentrations of Riboflavin, FMN and FAD determined via spectrophotometry; extinction coefficients of $12,200 \text{ cm}^{-1}\text{M}^{-1}$ for Riboflavin and FMN and $11,300 \text{ cm}^{-1}\text{M}^{-1}$ for FAD. Prior to running samples, a dilution series was prepared and run for each standard and a linear fit determined. With each batch of samples, 1 control for each standard is run in addition to a blank water and a blank methanol to determine any contamination or mobile phase interaction peaks.

MTHFR 1298 and 677 Status: Single Nucleotide Polymorphisms of MTHFR and NOS1 were determined using Taqman© Sample to SNP kits. Tubes containing buffy coat, white cells, are thawed and used for this analysis. Samples were vortexed and 5 μ L was added to an Eppendorf tube with 20 μ L of lysis solution. Tubes were vortexed and allowed to sit at room temperature for 5 minutes. Next, 20 μ L of DNA stabilizing solution was added to each tube before vortexing. Samples were centrifuged for 5 minute at 10,000 rpm.

A reaction mixture was prepared containing GTX master mix, the individual SNP assay and DNA-ase free water. In each RT-PCR reaction tube, 8.0 uL of the reaction mixture and 2.0 uL of sample were added. The plate was then vortexed and then centrifuged. RT-PCR analyzed fluorescence levels across 50 reactions; genotype is determined by plotting one allele's fluorescence strength against the other. In each batch of samples, one blank and one homozygous control for each allele are included for reference.

Nitrate/Nitrite: Using a Cayman Chemical colorimetric kit. Stored plasma samples were thawed and proteins were removed using a Millipore centrifugal filters. Kit was utilized per instructions using deproteinized supernatant. The prepared 96-well plate was read using a spectrophotometer at 540 nm.

Sodium Ingestion Blood Collection Appointments: On scheduled blood collection days, subjects arrived having fasted for at least 8 hours prior to scheduled appointment. Subjects sit in a phlebotomy chair and rest for 5 to 10 minutes prior to a certified phlebotomist inserting a 21 gauge butterfly needle attached to a catheter collection system. The needle is secured and one 10 mL EDTA tube and one 8 mL serum separator tube are collected and marked time -20. Another baseline collection is taken at -10 minutes and then at 0 minutes, the subject begins drinking a 475 mL bolus of 157 mM sodium chloride in water or water. Blood draws are taken again at 5, 10, 15, 30, 45 and 60 minutes following the beginning of sodium ingestion. Subjects were asked to complete drinking the bolus between the 0 and 5 min blood draws. Following the 60-minute blood draw, the catheter was removed and the patient check to ensure they were safe to stand and leave.

Sodium Ingestion Urine collection: Following the blood collection appointment, subjects were given a urine collection container and asked to collect all urine within the 8 hours following the bolus ingestion. Subjects were asked to keep the urine refrigerated and containers were placed in a 4C refrigerator immediately upon receipt in the lab. Containers were weighed prior to distribution and following collection to determine volume. One 50 mL tube of urine was stored in the minus 80 freezer until analysis.

Sodium and Potassium via Flame Photometer: A Cole Parmer flame photometer was used with pure propane from AirGas. A pressure of 30 PSI and flow rate of 0.4 mL/min were maintained using an air compressor at 11 PSI (6 liters per minute flow rate). Once flame was ignited, machine ran for 30 minutes with freshly prepared diluent running through aspirator. During this time, the blank dial was adjusted so machine reading was set to 0.00. Next a 10 ppm solution of sodium standard (reduced using the same batch of diluent) was aspirated for 20 sec while the coarse and fine dials on the machine were adjusted so the reading was at 100.00. The blank (diluent) was then aspirated again to ensure it read at 0.00 ± 0.01 . The two solutions were repeated until accuracy was verified. Each time a solution is aspirated, it is held for 20 seconds, then a blank is run after 10 seconds of no aspiration. Next a dilution series of sodium standards were aspirated starting with 5ppm, then 2.5ppm then 1ppm then 0.1ppm. For each standard, the read out was recorded to create a linear correlation between light omission and concentration (ppm).

Plasma and urine samples were thawed and diluted 1:200 with the same batch of diluent used for the standards. These samples were then run on the flame photometer and the light omission recorded. Concentration was calculated by determining the ppm via the standard equation, multiplying by 200 (to account for the dilution) and then

converting 1 ppm = 0.0435 mmol/L. The same process and calculations are completed

with potassium using 1 ppm = 0.0256 mmol/L.

A3: Method Validity

HPLC Riboflavin Assay: 100 nmol concentrations of standards were run before each batch of 30 samples. Two lab controls, J and M, were run with each batch of unknown samples (Table 9).

	Peak Area		
	FAD	FMN	Riboflavin
	520.1	255.4	157.2
	557	257.1	172.7
	600.9	250.3	189.1
	485.2	262.3	206.6
Avg	540.8	256.3	181.4
SD	49.6	4.9	21.3
CV	9%	2%	12%

	Plasma Control J			Plasma Control M		
	FAD	FMN	B2	FAD	FMN	B2
	51.2	4.7	6.8	92.8	5.4	27.9
	20.3	21.8	11.17	89.4	29	22.1
	80.3	8.8	10.9	111.3	19	19.6
	69.5	7.2	7.1	119.2	35.4	17.7
Avg	56.7	12.6	9.723333	106.6333	27.8	19.8
SD	31.9825	8.007496	2.275881	15.43837	8.265591	2.206808
CV	56%	64%	23%	14%	30%	11%

Table 9: Riboflavin HPLC Assay Validity Data

EGRac Assay: Red blood cells from one subject from one blood draw were measured in each assay and the ratio of each run averaged to determine an acceptable range for all unknown samples (Table 10).

Trial	Red Blood Cell Control Ratio	Trial	Red Blood Cell Control Ratio		AVG	1.24649
1	1.288259	15	1.222066		STDEV	0.24516
2	0.895691	16	1.119968		CV	19.67%
3	1.277247	17	1.419661			
4	1.674123	18	1.501389	Acceptable Range		
5	1.21981	19	1.610823	1.0321	<-->	1.548
6	1.501772	20	1.355705			
7	1.303937	21	1.005304			
8	1.510187	22	0.862875			
9	1.174171	23	0.726486			
10	1.421165	24	1.250652			
11	1.180627	25	1.578743			
12	1.241325	26	1.033723			
13	1.419389	27	1.475351			
14	1.005123					

Table 10: EGRac Assay Validity Data

HPLC Homocysteine Assay: An internal standard of Cystamine was used in each run of 20 to 40 samples. In each batch the Coefficient of Variance provides the intra-assay variability and in these experiments ranged from 4 – 8%. An external standard of cysteine and homocysteine was added to each sample and provides the Inter-assay variability (Table 11). Plasma homocysteine reference range is 4-10 umol/L.

	Hcy	Cys
ES Peak Avg	121.98	122.02
ES Peak SD	6.32247314	23.1815803
ES CV	5%	19%

Table 11: Homocysteine HPLC Assay Validity Data

DNA SNP Analysis: Two samples were run 3 times to verify one as a CC genotype and one as TT genotype. These were run in each batch to provide a control for each genotype. These samples gave a reference number for the fluorescence level of each allele which was used as a threshold for verifying other samples genotypes.

Nitrite/Nitrate Assay: Standard solutions were provided, with the assay kit, containing nitrates/nitrites which we diluted to create a curve (0uM – 25uM). We also included a blank and some lab controls on the 96 well plate with unknown samples. Average CV, 8.7%, is an average of the CVs from all the lab controls and standard curve values (each run in duplicate or triplicate) (Table 12).

				Avg	SD	CV
0 uM	0.280	0.247		0.263	0.024	9.0%
5 uM	0.530	0.482		0.506	0.034	6.7%
10 uM	0.850	0.787		0.819	0.045	5.5%
15 uM	1.066	0.995		1.031	0.050	4.9%
20 uM	1.302	1.140		1.221	0.115	9.4%
25 uM	1.548	1.306		1.427	0.171	12.0%
Blank	0.183	0.152		0.168	0.022	13.1%
AH	0.375	0.301	0.318	0.332	0.039	11.6%
JM	0.507	0.483	0.524	0.505	0.021	4.1%
MM	0.285	0.344	0.241	0.290	0.052	17.8%
Plasma UTAK	0.275	0.246	0.276	0.266	0.017	6.5%
Serum UTAK	0.254	0.257	0.240	0.250	0.009	3.6%
Overall Assay CV						8.7%

Table 12: Nitrate/Nitrite Assay Validity Data

Sodium/Potassium Assay: The sodium and potassium measurements on flame photometer were done using a calibration curve. The same dilutions were used to establish the fluorescence curve for all samples in this dissertation. Serum Sodium reference range is 135 – 145 mmol/L. (Table 13).

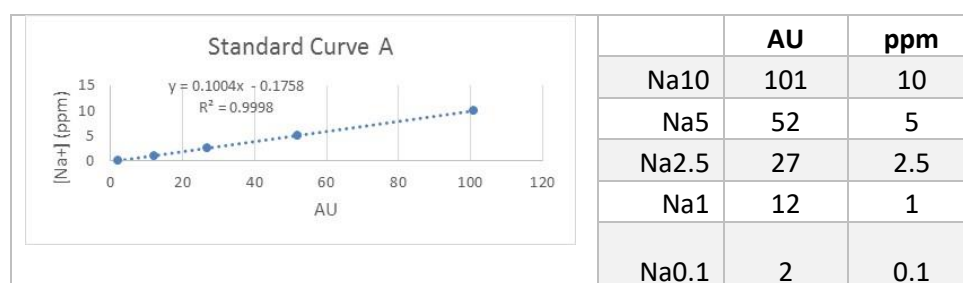


Table 13: Sodium Flame Photometer Assay Validity Data

A4: Blood Pressure Recording Form

Date:					Date:				
Trial #:					Trial #:				
Subject:					Subject:				
Time	Time Post	Systolic	Diastolic	Pulse		Time Post	Mood	Physiology	Cravings
	-20					-20			
	-10					-10			
	0					0			
	10					10			
	20					20			
	30					30			
	40					40			
	50					50			
	60					60			
	70					70			
	80					80			
	90					90			
	100					100			
	110					110			
	120					120			
Time	Time Post	Systolic	Diastolic	Pulse		Time Post	Mood	Physiology	Cravings
	-20					-20			
	-10					-10			
	0					0			
	10					10			
	20					20			
	30					30			
	40					40			
	50					50			
	60					60			
	70					70			
	80					80			
	90					90			
	100					100			
	110					110			
	120					120			

A5: Acute BP and Anticipatory Response Study Questionnaire

Subject #_____

Please answer the questions to the best of your knowledge and add any necessary notes.

1. **What is your current height? weight?**
2. **What is your age?**
3. **What is your ethnicity?**
4. **Do you have a history of any medical conditions (if yes, please list)?**
5. **Do you take any medications (if yes, please list)?**
6. **Have you been diagnosed with hypotension (low blood pressure) or hypertension (high blood pressure)?**
 - a. *Yes (please answer the following questions)*
 - i. **What symptoms (if any) caused you to first seek treatment?**
 - ii. **What medications do you use for this condition?**
 - b. **No**
7. **Do you smoke?** Yes / No **Have you ever smoked?** Yes / No
8. **On an average day, what time do you wake up?**
9. **On an average day, what time do you first eat a meal?**
10. **How many times per week do you participate in at least 30 minutes of exercise?**
11. **In a given day, how much water do you typically drink?**
12. **Indicate which foods below you like and of those how much and how often you eat them.**

	Do you like it? (Y/N)	How much (per month)?	How Often (per month)?
Potato Chips			
Pickles			
Pretzels			
Canned soup			
Table Salt			
Cheese			

TV dinners			
Pasta Sauce			
Cold Cereal			
Beef Jerky			
Ketchup			
Salad Dressing			
Pizza			
Cakes/muffins			

A6: MTHFR Resting and Acute BP Study Questionnaire

Please answer the questions to the best of your knowledge and add any necessary notes.

BP1: _____

BP2: _____

BP3: _____

13. What is your age?

Ethnicity/race?
Weight?

Sex?
Height?

14. Do you have a history of any medical conditions? (if yes, please list)?

15. Do you take any medications? (if yes, please list)

16. Have you been diagnosed with hypotension (low blood pressure) or hypertension (high blood pressure)?

a. If yes, what symptoms (if any) caused you to first seek treatment?

b. What medications do you use for this condition?

17. Do you smoke? Yes / No

Have you ever smoked?

Yes / No

18. Do you use any products with vitamin or mineral supplement/fortification (ex: pills, powders, vitamin drinks?)

a. How often do you take these?

b. What amount?

c. What are the brand names of these products?

19. Indicate how much and how often you eat the following foods:

	in the last 24 hours?	in the last week?
Enriched/Fortified cereals		
Enriched/Fortified Breads		
Yeast		
Tempeh		
Almonds		
Other Nuts		
Sesame Seeds		
Turkey		
Beef		
Pork		
Liver		
Fish		
Orange Juice		
Milk		
Yogurt		
Eggs		
Soybeans		
Black Beans		
Kidney Beans		
Navy Beans		
Lentils		
Pinto Beans		
Garbanzo Beans		
Potatoes		
Asparagus		

	in the last 24 hours?	in the last week?
Kale		
Spinach		
Turnip Greens		
Broccoli		

20. What have you had to eat this morning?

A7: Relationship between MTHFR 1298 and BP

There was not an association between MTHFR1298 and SBP ($p = 0.08$) or DBP ($p = 0.36$) (Table 14).

	SBP (mmHg)	DBP (mmHg)	n
AA	111.4 ± 9.6	71.4 ± 9.9	46
AC	117.0 ± 13.6	74.8 ± 11.8	35
CC	111.1 ± 11.1	71.6 ± 10.4	8

Table 14: MTHFR Resting BP Cohort Study MTHFR 1298 Genotype Associations

A8: Relationship between NOS3 and BP

There was not an association between NOS3 and SBP ($p = 0.24$), or DBP ($p = 0.98$) (Table 15).

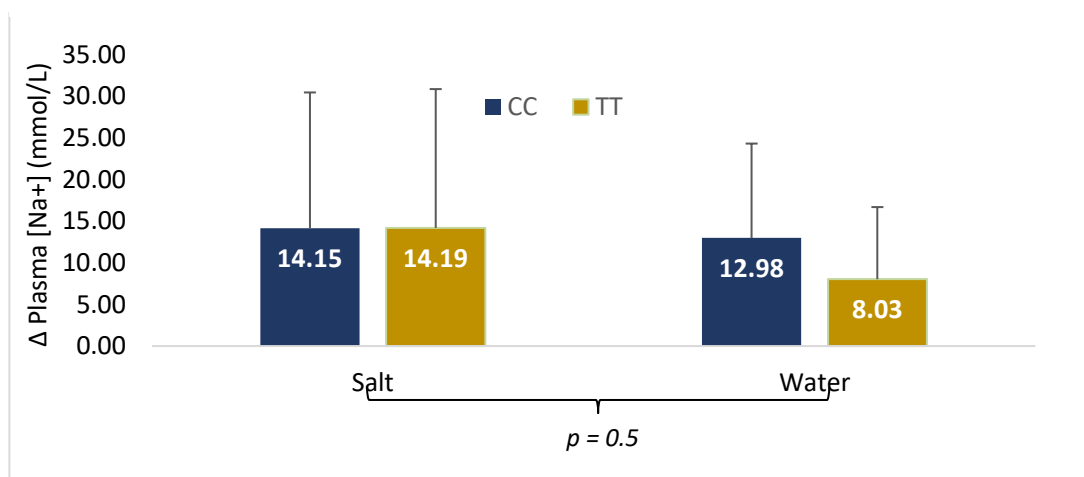
	SBP (mmHg)	DBP (mmHg)	n
1	114.0 ± 11.5	72.7 ± 12.3	43
2	112.9 ± 11.2	72.4 ± 8.5	36
3	110.6 ± 9.2	71.8 ± 3.9	5

Table 15: MTHFR Resting BP Cohort Study NOS3 Genotype Associations

A9: MTHFR Acute BP Study Plasma Sodium and Potassium Post Salt Ingestion

We measured plasma sodium post ingestion of salt and water to see fluctuations in blood sodium levels that may relate to BP changes. Plasma sodium in both groups increased post ingestion of both water and sodium. Baseline average for CCs was 112.5 ± 10.3 mmol/L and for TTs was 116.2 ± 7.7 mmol/L. Max plasma sodium following water ingestion was 113.0 ± 7.8 mmol/L for CCs and 116.7 ± 6.6 mmol/L for TTs. Max plasma sodium following salt ingestion was 111.9 ± 12.8 mmol/L for CCs and 115.7 ± 9.0 mmol/L for TTs. Despite the change in plasma sodium, from baseline and max, there was not a statistical difference between these values ($p = 0.38$). There were also not statistical differences between CCs and TTs ($p = 0.5$) (Figure 44).

Figure 44: MTHFR Acute BP Study Plasma Sodium Post Salt Ingestion



Left two bars are following salt ingestion and right two bars are following water ingestion

Though plasma sodium was not significantly different, we considered the relationship between sodium and potassium. At baseline and maximum ratio of sodium to potassium post ingestion, there were no differences between genotypes groups ($p = 0.28$). Change in the ratio of sodium/potassium from baseline to max was similar between genotype groups following sodium ingestion compared with water ($p = 0.34$). There were no differences between ratio following salt v water ingestion ($p=0.4$) (Table 16).

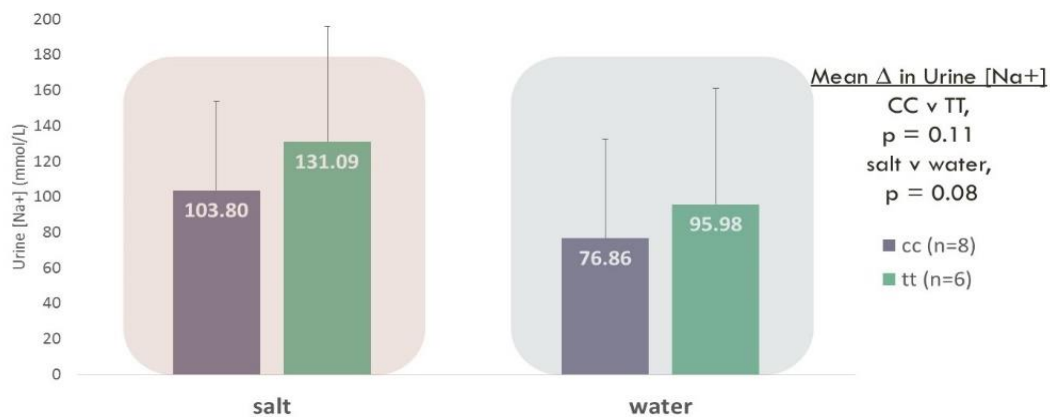
	Na (nmol/L)			K (nmol/L)			Na/K		
	Start	Max	Delta	Start	Max	Delta	Start	Max	Delta
TT NaCl	115.69	129.88	14.19	21.56	23.15	3.90	5.41	5.17	-0.24
TT Water	116.71	124.74	8.03	22.30	32.30	12.48	5.78	3.98	-1.86
CC NaCl	111.93	126.08	14.15	21.89	28.89	7.00	5.44	4.52	-0.92
CC Water	112.99	125.97	12.98	22.47	36.05	13.58	5.09	3.75	-1.34

Table 16: MTHFR Acute BP Study Plasma Sodium/Potassium Data

A10: MTHFR Acute BP Study Urine Sodium Post Salt Ingestion

We measured urine sodium excretion to understand if there were differences in rate of sodium excretion that may impact BP. Urine sodium excretion in both genotype groups was greater following sodium ingestion compared with water ingestion, though not statistically significant. In both cases, urine sodium excretion was greater in the TTs than the CCs, 131.09 v 103.8 nmol/L following NaCl ingestion and 95.98 v 76.86 nmol/L following water ingestion ($p=0.11$). Potassium excretion was not significantly different between TTs and CCs: 16.17 v 18.04 nmol/L following NaCl ingestion and 19.42 v 26.54 nmol/L following sodium ingestion ($p = 0.34$)

Figure 45: MTHFR Acute BP Study Urine Sodium Post Salt Ingestion



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Discerning the Effect of Salt, Riboflavin and MTHFR on Acute Blood Pressure Response

by MELISSA ANNE MURPHY

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