AMERICAN UNIVERSITY OF BEIRUT

DIETARY PHOSPHATE REDUCES HYPERTENSION, INFLAMMATION AND END ORGAN DAMAGE

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Pharmacology and Toxicology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
August 2021

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ACKNOWLEDGEMENTS

For the Lord give wisdom; from his mouth come knowledge and understanding (Proverbs 2:6).

First and foremost, I would like to express my deep and sincere gratitude to my thesis advisor Dr. Hana El Itani, who is not just a great mentor but also a great person inside and out. I would like to thank her for the continuous support, motivation, advice and patience throughout my Master's journey. I am grateful to her for welcoming me to her laboratory to work on my thesis project and for her continuous guidance and encouragement to become the dedicated and passionate student I am now.

Also, I would like to thank Dr. Omar Obeid for his contribution, support, and help without you, this project would not have been realized. I would like to express my warm gratitude to the committee members, Dr.Sabra, Dr.Abou Kheir, and Dr.El-Sabban, for their helpful tips, constructive advice and support that helped in shaping my professional journey at AUB.

I am extremely grateful to Ms. Amira Bekdash for the help, support, and knowledge that she provided me during this journey. I very much appreciate your time and efforts to make me learn and master all the Laboratory techniques and analysis while facing several bumps starting from Thawra to Covid-19 to the economic crisis, without you, the completion of my thesis would not have been possible.

A special thanks to my colleague Diana Ismail, who I met by chance on the first day of graduate school and never thought that we would become best friends more like sisters. Thank you for always being there, for all the support, for all the motivation, for all the care, and especially for coping with all my mood swings. I would like to thank you for always supporting me to give my best and not quit in the middle of the journey, I will be forever thankful.
I also wish to acknowledge my lab mates for the help and experience I gained from them, especially Dr.Zeina Radwan, Dr.Dina Maaliki, and my colleagues Aya Al Saidi, ,Karna Jabotian, Ibrahim Al Zaim, Hala Jarrah and Mohammad Labban for their endless support and motivation.

My appreciation also goes out to all my friends, to all my relatives, to my second family, the 503 Red Cross Family, and to my number one supporter, my boyfriend Michel, for always being there showering me with love and support.

Last but not least, I would like to express my profound gratitude and thankfulness to Mum, Dad, Christine, and Wadih for always cheering me up, motivating me, challenging me, and encouraging me, I love you so much.

ABSTRACT
OF THE THESIS OF

Yara Elie El Atie for Master of Science
Major: Pharmacology and Therapeutics

Title: Dietary Phosphate Reduces Hypertension, Inflammation, and End Organ Damage

Hypertension remains an increasing global health burden. Previous research has shown that dietary phosphate can modulate cardiovascular disease (CVD), yet the precise mechanism of how dietary phosphate contributes to blood pressure (BP), renal injury, and CVD remains unclear. Thus, we hypothesized that phosphate (P) reduces BP, inflammation, and end-organ damage. To this end, male C57bL/6 mice were either infused with four weeks of saline or Angiotensin II (490ng/kg/min) using a subcutaneous mini-osmotic pump. Over these 4 weeks, mice were fed a control diet for 2 weeks, followed by 2 weeks of phosphate-rich diet (0.15%, control (0.3%), and 1.5%P). Tail-cuff BP recordings were measured throughout the study protocol. To assess kidney damage, albumin in 24-hour urine samples, Masson's trichrome staining, and DHE staining were performed. Flow cytometry was performed to examine immune cells infiltration in the kidney. Strikingly, our results show that control (0.3%P) and 1.5%P significantly reduced the SBP by 6.1 and 24.8 mmHg, respectively. In addition, CD4+T and CD8+T cell infiltration was dramatically reduced in the kidneys of the Angiotensin II-infused mice fed a 0.15%, control (0.3%P), 1.5%P diet compared to sham mice. Finally, Angiotensin II-infused mice fed a 0.15%, control (0.3%P), 1.5%P diet were protected from kidney glomerular damage, fibrosis, and ROS formation. We conclude that containing dietary phosphate mitigates hypertension and its end-organ damage.

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ABBREVIATIONS

ACC	American College of Cardiology
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin-Converting Enzyme 2
ACEI	Angiotensin-Converting Enzyme Inhibitor
ACR	Albumin-to-Creatinine Ratio
AGT	Angiotensinogen
AHA	American Heart Association
Ang I	Angiotensin 1
Ang II	Angiotensin 2
ARB	Angiotensin Receptor Blocker
AT_1R	Angiotensin II type 1 Receptor
AT_2R	Angiotensin II type 2 Receptor
BP	Blood Pressure
CKD	Chronic Kidney Disease
CNS	Central Nervous System
CVD	Cardiovascular diseases
DAMP	Damage Associated Molecular Patterns
DASH	Dietary Approach to Stop Hypertension
DBP	Diastolic Blood Pressure
DC	Dendritic cells
DHE	Dihydroethidium
ELISA	Enzyme-Linked Immunosorbent Assay
ENaC	Luminal Amiloride-Sensitive Epithelial Sodium Channel

eNOS	Endothelial Nitric Oxide Synthase
ESC	European Society of Cardiology
ESH	European Society of Hypertension
FGF23	Fibroblast Growth Factor 23
FMO	Fluorescence Minus One
GFR	Glomerular Filtration Rate
H&E	Hematoxylin and Eosin
IFN- γ	Interferon Gamma
IL	Interleukin
MedDiet	Mediterranean Diet
NCC	Sodium-Chloride Cotransporter
NFκB	Nuclear Factor Kappa B
NHE3	Sodium Hydrogen Exchanger 3
NK	Natural killer
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
PAMPs	Pathogen-Associated Molecular Patterns
PAS	Periodic-acid Schiff
PBS	Phosphate Buffered Saline
PNS	Parasympathetic Nervous System
PTH	Parathyroid Hormone
RAAS	Renin Angiotensin Aldosterone System
RBF	Renal Blood Flow
REN	Renin Gene

RNSA	Renal Sympathetic Nerve Activity
ROMK	Renal Outer Medullary K+
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SNA	Sympathetic Nerve Activity
TEM	T Effector Memory Cells
Th	Helper T cells
TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor Alpha

CHAPTER 1

INTRODUCTION

Cardiovascular diseases (CVD) are the leading causes of death in the United States and worldwide, where more than eight hundred thousand die annually [1]. According to the World Health Organization reports in 2019, 17.9 million occurred due to CVDS, of which 85% were due to stroke and heart attacks. Those deaths mainly occurred in middle to low income countries [2]. CVD is characterized by major health problems affecting the heart and the blood vessels, leading to mortality if not detected and treated. CVD include myocardial infarction, atherosclerosis, heart failure, cerebrovascular disease, coronary artery disease, peripheral artery disease, venous thrombosis, rupture of blood vessels, and cognitive impairment [3, 4]. The heart is one of the major organs in the human body responsible for delivering blood to all body parts. After each beat, the blood is pumped out of the left ventricle into the aorta and distributed to the body while exerting pressure on the walls of the blood vessels, known as blood pressure (BP). BP**,** measured in millimeters of mercury, is the ratio of the systolic pressure, the maximum blood pressure during which the heart muscle contracts to pump the oxygen-rich blood over the diastolic pressure, the minimum pressure recorded during which the heart muscle relaxes before the next contraction [5]. Blood pressure is regulated and maintained by specific mechanisms, regulators, and feedback pathways to reach homeostasis after any disturbance or change that might affect it, which will be explained.

In 2014, the Eighth Joint National Committee recommended that all the population aged above 60 years old maintain a BP less than 150/90 mmHg. In contrast,

the population aged less than 60 years old suffering or not from diabetes or CDV should have a BP less than 140/90 mmHg [6]. In 2017, an updated clinical guideline for the prevention, detection, evaluation, and treatment of hypertension was released by the American Heart Association (AHA) and the American College of Cardiology (ACC), setting the normal BP as less than 120/80 mmHg and the hypertensive BP as 130/80 mmHg or more [7]. On the other hand, the European Society of Cardiology (ESC), along with the European Society of Hypertension (ESH) specified hypertension as an increase in BP above 140/90 mmHg [8]. Many risk factors such as obesity, diet, race, age, sex, impaired estimated glomerular filtration rate (eGFR), impaired glucose regulation, dyslipidemia, and hyperuricemia, lead to the onset or development of hypertension [9]. Hypertension is associated with many health conditions, especially if left untreated, can lead to cardiovascular diseases mentioned earlier, mainly heart failure, heart attack, strokes, and coronary artery disease [4].

It has been shown in 2015 that out of the total deaths related to a systolic pressure of 140 mmHg or above, 41 percent were due to cardiovascular diseases and the rest due to chronic kidney disease [10]. Moreover, unhealthy diet such as high-salt or high-fat diet, tobacco use, lack of exercise, poor stress management, health conditions such as diabetes, obesity, dyslipidemia, hyperuricemia, impaired estimated glomerular filtration rate, resting heart rate (at least 80 bpm), genetic factors, either combined or alone can play an important role in the development or onset of hypertension [4, 9].

Hypertension is a significant public health epidemic with a high prevalence among different populations. The highest hypertension prevalence among adults aged above 25 years of age was in the lower-income countries such as the African region or the middle-income countries like Lebanon and the lowest in the high-income countries

like America [4]. Among the U.S. adults, the crude prevalence of hypertension in 2017-2018 was 45.6 percent, where 36.2 percent were given anti-hypertensive treatment, according to the 2017 ACC/AHA guidelines. Out of those adults with hypertension, 51 percent were men, and 39.7 percent were women [11, 12] . A meta-analysis done by Song et al. discovered that the prevalence of hypertension among children has increased in the last two decades reaching in 2015, 4.32 percent for six-year-old children, 7.89 percent for 14 years old children, and 3.28 percent for 19 years old children [13]. In Lebanon, a study was done to scrutinize the prevalence of hypertension in the Lebanese population discovered that 399 out of 1362 adults aged between 18 and 95 years had hypertension [14].

1.1. Hypertension

1.1.1. Renin Angiotensin Aldosterone System (RAAS)

In the human body, blood pressure and fluid balance are tightly controlled and maintained by the hormonal axis of the Renin-Angiotensin-Aldosterone System [15]. RAAS is essential for human survival since it maintains normal extracellular fluid volume and arterial pressure and tightly regulates blood plasma, interstitial fluid, lymph, and water to sustain a normal vessel tonicity and a normal kidney and heart function [16]. Renin, secreted from the juxtaglomerular cells of the kidneys, mediates the rate-limiting hormonal enzyme leading to the production of the RAAS' end product Angiotensin II (Ang II), responsible for keeping blood pressure and electrolyte homeostasis [17]. In the 18th century, renin, also known as angiotensinogenase, was discovered when a rabbit was injected with another rabbit's fresh kidney saline showed an increase in his arterial pressure. The gene REN1 encoding for renin is located on the

human chromosome 1 at the locus 1q32 [18]. Normally, renin is secreted in an inactive form of prorenin by the kidney when the blood pressure is low, or the sodium chloride concentration is low, or the beta-1 adrenoreceptors are activated. Then prorenin is converted into renin either non-proteolytically when binding to prorenin/renin receptor or proteolytically in the kidney by Cathepsin B or Neuroendocrine convertase 1 [16].

As shown in Fig 1 below, following renin secretion, the liver globulin protein angiotensinogen (AGT) is hydrolyzed into angiotensin I (Ang I). Then, Ang I is cleaved by the angiotensin-converting enzyme (ACE) into an intracrine, autocrine, and paracrine hormone Ang II, which can cross-talk with many-body systems by increasing the expression of prostaglandins, and angiotensin III. This cleavage occurs in the kidney epithelial cell, lung capillaries, and endothelial cells. Ang II has a short half-life of 30 seconds, so if it does not bind to its specific receptors, it can be converted into angiotensin III via the enzyme aminopeptidase A on red blood cells or via the enzyme angiotensin-converting enzyme 2 (ACE2), which is critical for renal homeostasis [16]. There are two types of receptors from the family of the G-protein coupled receptor where Ang II can bind; the angiotensin II type 1 receptor (AT_1R) present in humans and rodents, and the angiotensin II type 2 receptor (AT_2R) present in humans predominantly during development, and its expression decreases in adult age. When activated, each receptor will lead to specific regulations and functions opposite to each other [19, 20].

Classically, RAAS actions are mediated by the binding of Ang II to AT_1R, leading to a cascade of events. First, Ang II increases BP and the pulsation speed of the heart by vasoconstriction through constricting the smooth muscle of the blood vessels and promoting the release of prostaglandins. Second, it affects the central nervous system by acting on the hypothalamus, inducing the thirst reflex's stimulation, and

targeting the pituitary gland to secrete vasopressin or the antidiuretic hormone that lessens urination. Third, lipogenesis, adipose inflammation, and insulin resistance are also promoted by Ang II. Last, Ang II is responsible for releasing aldosterone by the adrenal cortex, which increases the BP and sodium retention through the kidney proximal tubules (fig.1) [16].

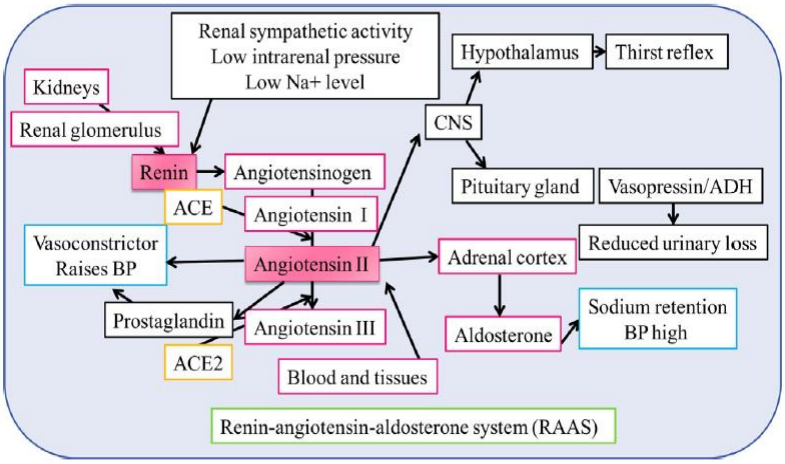

Figure 1. The fundamental mechanisms of RAAS. Renin released from the kidneys leads to the formation of Ang II causing elevated BP, vasoconstriction and sodium retention [16]

On the other hand, the counter-regulator RAAS pathway is mediated by the binding of Ang II to AT_2R and by ACE2/Mas receptor/Angiotensin (1-7) axis [19, 21]. Even though the expression of AT_2R is dominant during development, its expression is increased through pathological states such as diabetes, cardiac failure and fibrosis, renal failure, and atherosclerosis. ACE2, a neutral- and prolyl- endopeptidases, is responsible for limiting the amount of Ang II substrates by increasing the conversion of Ang I into angiotensin (1-9) and limiting Ang II availability by changing it to angiotensin (1-7), which bind to AT_2R or Mas receptor resulting in a decreased binding of Ang II to AT_1R and opposing its effects on elevating BP and on increasing sodium retention. Therefore, ACE2/Mas receptor/Angiotensin (1-7) axis activation will promote vessel dilation,

sodium excretion, anti-inflammatory, and anti-proliferative effects self-protection against diseases concerning the heart, kidneys, liver, bones, and metabolism [21, 22].

One of the main effector products of RAAS responsible for increasing the BP and plasma sodium level is aldosterone. Aldosterone is a steroid hormone secreted from the zona glomerulosa of the adrenal cortex when Ang II and potassium (K^+) levels are increased [23]. This hormone is synthesized from cholesterol after a series of enzymatic reactions mediated by the gene CYP11B, encoding the enzyme aldosterone synthase. Aldosterone binds to the mineralocorticoid receptors located in the cytosol of epithelial cells in the renal collecting duct, in the colon, and parotid gland to influence the retention of sodium (Na^+) and excretion of potassium. This binding exerts conformational changes to the hormone, making it an active complex that binds to responsive elements, activating or suppressing gene transcription. In the epithelia, aldosterone has two phases of action the early phase from one to six hours activating ion channels and signaling proteins and the late stage after 6 hours affecting gene and protein expressions such as the serine-threonine kinase (SGK1), the Kirsten Ras GTP-binding protein-2A (Ki-RasA) and Corticosteroid hormone-induced factor (CHIF). These proteins are known to increase the expression of the sodium ion channels in the epithelial cells, especially in the collecting duct in the kidney [24]. Along the apical membrane, sodium is reabsorbed from the urinary space of the collecting into the plasma driven through the luminal amiloride-sensitive epithelial sodium channel (ENaC) facilitated by an electrochemical gradient followed by water reabsorption eventually [25]. ENaC is composed of 3 subunits $\alpha\beta\gamma$ when aldosterone is present, the α subunit is upregulated in the kidney, and the $\beta\gamma$ subunits are upregulated in the colon.

While across the basolateral membrane of the epithelial cell, sodium enters the cell, and potassium exits it via the Na^+/K^+ ATPase channel [24]. In addition to this channel, potassium (K^+) homeostasis is another fundamental ion regulated by aldosterone. When the concentration of K^+ is elevated in the plasma, aldosterone secretion will be stimulated to activate the K^+ channels allowing the secretion of potassium into the distal tubules of the kidney, eventually liberating K^+ in urine outside of the body. The channel responsible for this secretion is the renal outer medullary K^+ (ROMK) located apically on the distal part of the nephron. They are 3 types of ROMK that differ slightly in structure and location, ROMK1 has two targets for protein kinase C (PKC) phosphorylation at the serine residues at the N-terminus, the cortical connecting duct.

In contrast, ROMK2 lacks these sites since it has a truncated protein found at the distal convoluted tubule or the connecting tubule, and ROMK3 is targeted for PKC phosphorylation threonine residue on the extended N-terminus also located at the distal convoluted tubule [26, 27]. Another aldosterone-sensitive K^+ channel located at the distal nephron is known as the Ca2+ activated K^+ channel (BK). BK stimulated by an increase in plasma K^+ is also a channel responsible for potassium secretion into the urine to be expelled from the body to maintain potassium homeostasis [26].

Accordingly, RAAS is a pivotal system in the human body to maintain blood pressure and electrolytes homeostasis, especially sodium and potassium . That is why Ang II formation and elimination should always be balanced and under control via RAAS to prevent any physiological conditions related to blood pressure, renal homeostasis, and cardiovascular health. Thus, the suppression of the expression of enzymes responsible for the metabolism of Ang II or an increase in ACE availability

will lead to an overexpression of Ang II in the plasma or tissues, playing a critical role in hypertension pathology [28]. To this extent, three classes of RAAS inhibitors can be used to treat hypertension by altering the effect of Ang II, leading to a reduction in BP, proteinuria, and inflammation. The first class Aliskiren, a direct inhibitor of renin, exerts its effect on the first step of RAAS, blocking the conversion of AGT to Ang I, reducing the formation of all the downstream products of RAAS especially Ang II. The second class comprises the ACE inhibitors (ACEI), which stop the conversion of Ang I into Ang II, limiting the secretion of aldosterone and vasopressin. The angiotensin receptor blocker (ARB), which blocks the AT_1R, diminishes the binding and aldosterone activity. The third class is the aldosterone antagonists, available as spironolactone and eplerenone, that bind to the mineralocorticoid receptor inhibiting aldosterone's action, hence preventing sodium retention at the level of the nephron's collecting duct in the kidneys [29, 30]. These classes can be given alone or in combination or in addition to some diuretics such as thiazide diuretics, loop diuretics, potassium-sparing diuretics, depending on the pathological stage of each patient to reach a better outcome in controlling hypertension and in reducing the cardiovascular risks [31].

1.1.2. Diet

Dietary Intake has been shown to substantially impact the onset of hypertension and eventually lead to CVD development [32]. In this context, a meta-analysis was done to display the correlation of several kinds of food with the risk of developing hypertension. They discovered a positive correlation between red meat (100g/d), processed meat (50g/d), sugar-sweetened beverages (250ml/d), and the risk of

hypertension. However, a negative relationship was observed between whole grains (30g/d), fruits (100g/d), dairy (200g/d), nuts (28g/d), and the development of hypertension [33]. Besides, obesity and a western diet is characterized by excess sodium, sugars (fructose, sucrose, and glucose), and saturated fats intake with a low amount of fruits, vegetables, whole grains, and omega-3 fatty acid were shown to have a strong relation with CVD and increased BP. In contrast, a moderate intake of vegetables, phosphorus, calcium, magnesium, fibers, non-heme iron, and unsaturated fats was shown to have an indirect relation with BP [34, 35]. All this must be controlled and managed by everyone prone or not to develop hypertension. It can start with preventing obesity by decreasing weight and BMI since a one-kilogram decrease in body weight is manifested by 1 mmHg decrease in BP [34].

Many flourishing and beneficial diets can be adopted to maintain a healthy lifestyle and avoid serious health problems. Starting with the Dietary Approach to Stop Hypertension (DASH) diet implemented by the National Institute of Health (NIH) in the 1900s which promotes the introduction of fruits, vegetables, low-fat dairy, healthy carbs (whole grains, beans), good fats (avocado, nuts, omega 3 fatty acids), micronutrients and lean meat and the reduction of sodium, fats and processed food [36]. A study compromising 412 participants with a mean BP of 135/86 mmHg has demonstrated that adherence to the DASH diet for one week can significantly reduce the systolic BP by 4.36 mmHg and the diastolic BP by 1.07 mmHg [37]. Generally, the DASH diet can lower the resting systolic and diastolic pressure by 11 and 5 mmHg [34]. Individuals adopting this lifestyle have a chance to reduce their risk of stroke by 20% and CVD by 13% [38]. Another benefit of adhering to the DASH diet is its protective effect slowing the decline of the estimated glomerular filtration rate and the risk of chronic kidney

disease since it has been demonstrated to interact with RAAS. This interaction results in a natriuretic-like effect manifested by vascular and hormonal responses augmenting the effect of the ACE inhibitor in individuals with hypertension [39, 40]. Even though the DASH diet is vital to reduce the risk of hypertension and CVD, yet adherence to it remains poor. According to the National Health and Nutrition Examination Survey (NHANES), the adherence to DASH among the US population from 1988 to 2004 was less than 1%. From 2007 to 2012, the score of adherence was approximately 2.6 out of 9 [41].

Another diet that has also been proven to lower CVD and hypertension is the Mediterranean diet (MedDiet). The MedDiet is also composed of fruits, vegetables, nuts, olive oil, fish, potatoes, beans, limited red and processed meat, and moderate dairy foods [34, 42]. A study performed on 3775 adults between 2013 and 2015 taken from the Hellinic National Nutrition and Health Survey (HNNHS) discovered that a firmer adherence to the MedDiet could decrease the possibility of hypertension by 36% [43]. The last diet that can also reduce the CVD and hypertension risk is vegetarian, which can be all plant (vegan), or plant-based with some dairy products (Lacto-avo-vegetarian), or plant-based with fish (pescovegetarian). A meta-analysis showed that adherence to this type of diet provided a significant decrease in the systolic BP by 2.66 mmHg and the diastolic BP by 1.69 mmHg [44]. To end, adopting a healthy diet is a critical risk factor for developing CVD, hypertension, diabetes, cancer, and obesity. This table from Locke et al. summarizes the diets we elaborated on previously, their composition, risk benefits, and special considerations [45].

Table 1. Summary of Common Dietary Patterns [45]

Diet	Includes	Restricts	Health Benefits
Dietary Approaches to Stop Hypertension	52% to 55% carbohydrates, 16% to 18% proteins, and 30% total fat; rich in fruits, vegetables, whole grains, and low-fat dairy products.	Limits saturated fats, cholesterol, refined grains, and sugars; suggested sodium intake is less than 2,400 mg per day	Decreases CVD risk factors, blood pressure, obesity, and type 2 diabetes mellitus
Mediterranean	Fish, Monounsaturated fats from olive oil, fruits, vegetables, whole grains, legumes/nuts, and moderate alcohol consumption	Limits red meat, refined grains, and sugars	Decreases rates of type 2 diabetes, cancer incidence and mortality, age-related cognitive decline. CVD incidence and mortality, overall mortality, and obesity
Vegetarian or vegan	Plant-Based foods: grains, plant oils, nuts, seeds, legumes, fruits, and vegetables	Vegetarian diet avoids red meats, pork, poultry, fish, and possibly eggs; vegan diet excludes all animal products and in some cases, honey	Vegetarian diet decreases rates of type 2 diabetes and coronary artery disease. Vegan diet decreases rates of hypertension, obesity and CVD mortality.

1.2. Inflammation, Immunity and Hypertension

For decades till now, researchers have been trying to establish the link between hypertension and immunity, starting with the discovery of human vascular antigen in a hypertensive patient in 1961, to the discovery of an elevated serum level of IgG in hypertensive individuals in 1971, reaching in the 2000s the identification of how the adaptive and innate immune systems interact together during hypertension causing end-organ damage especially to the kidneys and blood vessels [46]. Figure 2 by Caillon et al. shows an excellent summary of how both systems interact together in response to hypertension [47]. Many factors like Ang II infusion, aldosterone, salt in the presence of specific genes, and endothelin-1 can induce the rise in BP by stimulating the brain to activate the sympathetic nervous system and suppress the parasympathetic nervous system. Over time, this minimal increase in BP causes organ damage to the vessels and the kidney via localized stress through the production of reactive oxygen species (ROS). This favors the production of damage-associated molecular patterns (DAMPS) and pathogen-associated molecular patterns (PAMPs), activating the innate immune since they are recognized by the Toll-like receptors (TLRs) on macrophages type 1 (M1), dendritic cells type 1 (DC1,) and natural killer cells (NK).

Also, the production of neoantigens enhancing the DC immunogenicity and endorsing the release of IL-6, IL-1β, and IL-23, promoting the proliferation of the T cells. These cytokines also activate the innate-like γδ T cells to activate the T effector cells, causing pro-inflammatory molecules. Therefore, innate immunity during hypertension can lead to inflammation indirectly via the recruitment of adaptive immunity [47, 48]. Once the effector T cells are activated, they infiltrate the kidney, blood vessels, heart, and brain and produce pro-inflammatory cytokines. IL-17A from

the CD4+ T helper 17, and the IFN-γ and TNF-α form CD8+ and CD4+ T Helper 1 and autoantibodies resulting in kidneys and vessel injury (endothelial dysfunction), further affecting the increase in BP. As inflammation is resolved, effector T effector cells undergo apoptosis, and a subset of effector memory T cells (TEM) will form in lymphoid organs such as the bone marrow. These cells are characterized by a hypertension-specific T cell receptor that is capable of recognizing the original neo-antigen. Thus, TEM cells are reactivated in response to a secondary stimulus, leading to their proliferation and tissue infiltration producing inflammatory cytokine, thus exacerbating kidney damage [47, 49]. It has been shown that CD8 knockout mice, but not CD4, infused with Ang II do not retain sodium and water and had blunted hypertension. These knockout mice were also protected from vascular injury. This further asserts that during hypertension, the accumulation of an oligoclonal population of CD8+ in the kidney is responsible for sodium and water retention contribution to elevated blood pressure [50]. However, during the process of hypertension and immune cell activation, some elements of the immune system try to stop or delay this inflammatory process, such as the T regulatory cells (Treg), the myeloid-derived suppressor cells (MDSCs), and the type 2 macrophages (M2) via the release of anti-inflammatory cytokine the IL-10. But this type of protection might not be as effective when the levels of Ang II are elevated because it promotes the decrease in the number

of Treg available and, therefore, decreasing its anti-inflammatory effects [47, 49].

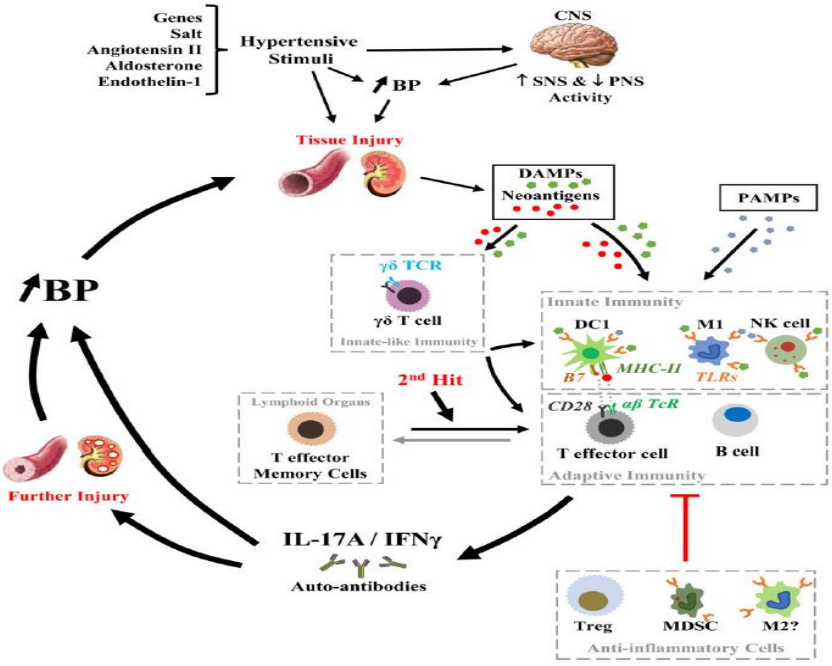

Figure 2. Summary of immune cells in hypertension. Hypertensive stimuli activate the CNS, increase BP and cause Tissue injury. All this will activate the adaptive and innate immunity releasing cytokines and causing further tissue injury and BP increase [47]

1.2.1. T Cells In Hypertension

As previously stated, once activated T cells, cytotoxic (CD8+) and helper (CD4+, Th 1), contribute to kidney and vascular damage leading to a further increase in BP. This was proven by studies in mice lacking all types of lymphocytes due to a deficiency in the recombinase-activating gene (RAG-1-/-) where they turned out to be protected against dysfunction in the endothelium, hypertrophy in vessels after being exposed to Ang II infusion, or deoxycorticosterone acetate-salt challenge (DOCA). The transfer on T cells and not B cells restored the hypertensive response and dysfunctions to those RAG-1-/- mice [51]. Once the mice BP is elevated following Ang II infusion, the DCs will release cytokines like IL-6 and IL-23 and increase its antigen-presenting

peptide (CD70, CD80, and CD86) that act as co-stimulatory signals for T cells. This will allow the proliferation and development of T cells, particularly CD8+, and the release of cytokines such as interleukin 17 (IL-17), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), increasing the incidence of hypertension, renal damage, vascular distress and end-organ damage [52]. To further assert that T cell is activated in hypertension and invade critical end-organ, Itani et al. were able to discover an increase in the infiltration of human T lymphocytes (CD3+ and CD4+) and leukocytes (CD45+) in the kidney, thoracic lymph nodes and thoracic aorta of a humanized mouse model treated with Ang II infusion. Also, they observed a high level of IL-17A produced by CD4+ cells and INF-γ produced by CD4+ and CD8+ [51]. Those CD8+ were oligoclonal cells invading the kidney after hypertension and causing sodium and water retention and vascular rarefaction [50]. The secretion of IL-17A by T cells throughout the hypertensive period was found to increase the sodium hydrogen exchanger three expressions through an Sgk1 pathway in human proximal tubule (HK-2) cells and increase the sodium-chloride co-transporter activity in an Sgk1/Nedd4-2 dependent pathway in mouse distal convoluted tubules. Thus, IL-17A-/- mice show a blunted hypertension response, abolished the activation of distal tubule transporters, and protected the mice from glomerular and tubular damage. This suggests that the inhibition of IL-17 may improve or delay renal dysfunction and end-organ damage in hypertension [53]. However, these effector T cells (CD8+ and CD4+) eventually undergo apoptosis, yet some of them will develop and become memory T cells (TEM) when their CD27 interact with the CD70 present on the activated DCs, so the lack of either CD27 or CD70 will stop the development of memory T cells. Some of the TEM will go back to the spleen and will be referred to as central memory having on their

surface the following markers $CD44^{hi}/CD62L^{hi}/CCR7+$, while the others stay as effector TEM in the periphery with surface markers $CD44^{hi}/CD62L^{lo}/CCR7-$. Thus the formation of these memory cells will make the T activation enhanced when re-challenged with a hypertensive stimulus leading to a faster release of IL-17A and INF-γ, causing disfunction to the kidney, increasing sodium and water retention, and increase the BP [54]. Finally, researchers have observed in the spleen an increase in the number and activity of another subset of T lymphocyte (innate-like) after Ang II infusion characterized by γδ T cell receptor, unlike the αβ T cell receptor that initiates the hypertensive immune response. These γδ T cells either act as antigen-presenting cells priming the activation of T cells or become activated by neoantigens triggering it to release pro-inflammatory cytokines such as IL-17A and IFN-γ, mediating the increase in BP, the vascular dysfunction, and the T cell activation [55].

1.2.2. The Effect of Cytokines on Hypertension

During hypertension, the pro-inflammatory immune cells (T cells and M1 macrophages) release cytokines that enhance the rise in BP, the vascular injury and renal damage such as interleukin 17A (IL-17), interleukin 6 (IL-6), interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF-α). Primarily, as seen in figure 3 by McMaster et al., T cells infiltrate the blood vessel and release IL-17 that act on the smooth muscle cells increases the level of the endothelial nitric oxide synthase (eNOS), chemokine, and collagen resulting in the suppression of the bioavailable nitric oxide (NO) causing vasoconstriction, vessel stiffness, and more recruitment of immune cells leading to vascular injury [56]. IL-17 also increases the sodium reabsorption in the kidney via regulating the sodium-chloride cotransporter (NCC) and the sodium

hydrogen exchanger 3 (NHE3) in the distal and proximal tubules [53, 57]. Secondarily, the T helper 1 cells go into the cortex and medulla of the kidney, where they are activated and produce IFN- γ and IL-6, increasing the production of AGT. AGT will eventually become Ang II. Ang II upregulates the transport channels NHE3 and NCC, increasing sodium and water retention. In conjunction with this, an increase in renal injury and fibrosis and renal ROS resulted in renal dysfunction exacerbating hypertension (Fig 3) [56, 58]. Thirdly, TNF-α not mentioned in the figure has an essential role in decreasing the renal blood flow (RBF) and the glomerular filtration rate (GFR) through vasoconstriction and exert its natriuretic effect through the TNF-α receptor 1 [59]. Upon binding the TNF-α to this receptor, the activation of survival and death pathways, c-Jun N-terminal kinases, NADPH oxidase, and nuclear factor kappa B (NFκB) occurs. NADPH oxidase and NFκB lead to the expression of chemokines and adhesion molecules, contributing to vascular remodeling and sodium retention. Also, TNF-α causes the inhibition of eNOS, altering the ability of the endothelium to release NO, therefore, impairing vasodilation and promoting vasoconstriction [56]. However, not all cytokines are pro-inflammatory since IL-10 is known for its anti-inflammatory effects ranging from the inhibition of NFκB to suppressing the cytokines, as mentioned earlier release. IL-10 produced by the T helper 2 cells, Tregs, monocytes and mast cells reducing BP, vascular inflammation and endothelial dysfunction [56, 57].

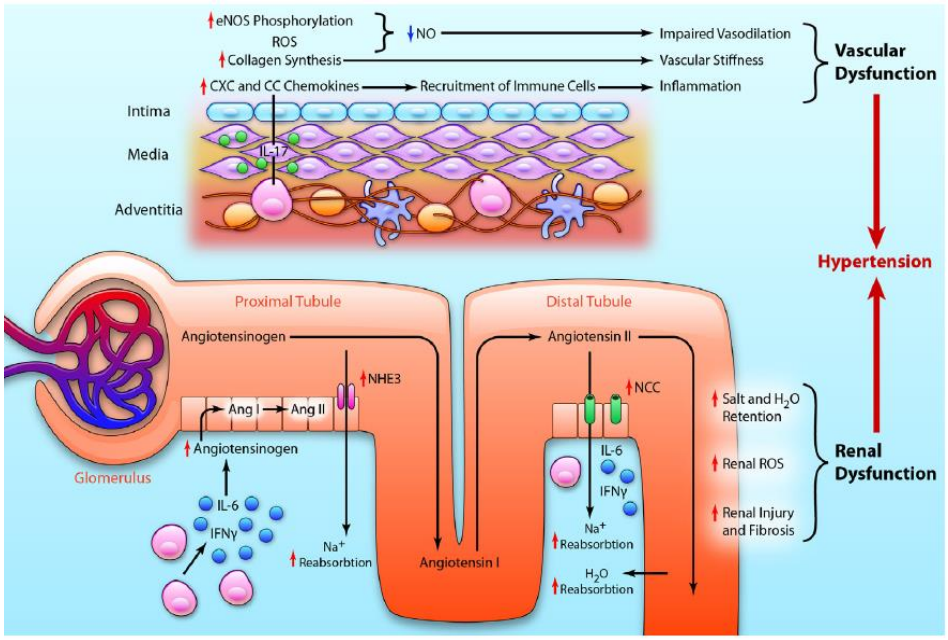

Figure 3. Cytokines and end-organ dysfunction in hypertension. T cells infiltrate the vessels and release IL-17A which acts on the smooth muscles increasing eNOS, collagen and chemokines production leading to impaired vasodilation, vascular stiffness and inflammation contributing to vascular dysfunction. On the other hand, T cells infiltration in the kidneys release cytokines (IL-6, IFN-γ) activating the formation of Ang II from AGT increasing sodium reabsorption. In addition, T cells activation increase renal ROS formation, renal injury and fibrosis. Therefore, both vascular and renal dysfunction caused by T cell activation and cytokine release lead to hypertension [56]

1.2.3. The Sympathetic Nervous System Is Proinflammatory

One of the hallmarks in hypertension is the over-driven activity of the sympathetic nervous system (SNA), characterized by the tonicity control of vessels and further stimulates the spleen, the bone marrow, the thymus, and the lymph nodes via the sympathetic noradrenergic nerve fibers. This stimulation will activate various immune cells since they express on their surface α and β adrenergic receptors. This SNA is dramatically increased during Ang II infusion, or DOCA-salt challenge, and in genetic spontaneous hypertensive rats [60]. The excessive stimulation of SNA will eventually lead to cardiovascular diseases, vascular damage, and kidney injury contributing to

mortality and morbidity. In hypertension, the activation of RAAS stimulates the sympathetic system to send signals via the adrenergic nerves to the bone marrow and spleen. They activate the innate immune cells, which in turn either directly infiltrate and inflame the kidneys, the heart, and the vessels, or release cytokines. This will activate the adaptive immunity, or home toward the central nervous system (CNS) resulting in positive feedback enhancing the SNA directed to the vascular tissue, heats, and kidney exacerbating hypertension, inflammation, and end-organ damage [61]. However, the parasympathetic system (PNS) has a protective activity through an anti-inflammatory, an immune response known by cholinergic anti-inflammatory reflex where after receiving vagal afferent signals, the PNS reflexively send efferent vagal signals to negatively modulate the production of the cytokine in the spleen by stimulating the α7-nicotinic cholinergic receptors (nAchR) as seen in figure 4 by Singh et al. [61]. It is noteworthy that researchers discovered that when there is renal denervation, there is inhibition of DC activation in the kidney and less infiltration of immune cells in the kidney; therefore, this denervation stops the end-organ damage [60]. This lead to the discovery that the elevated renal sympathetic nerve activity (RSNA) is crucial for the contribution to high blood pressure in three ways: first via increasing the tubular reabsorption of water and sodium, second via reducing the GFR and RBF, third via releasing renin and activating RAAS eventually leading to end-organ damage [62].

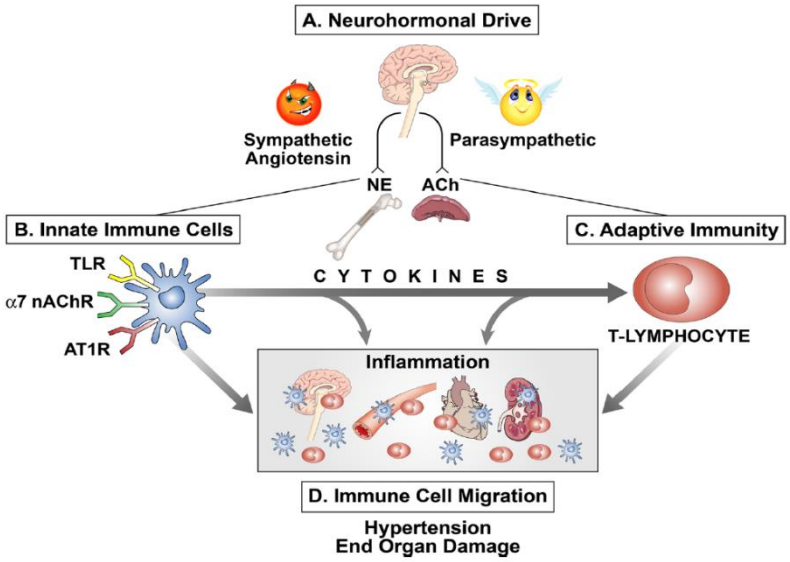

Figure 4. Interactions between autonomic and immune systems. The neurohormonal drive through the sympathetic and parasympathetic activates the innate and adaptive immunity causing inflammation and immune cell migration to the brain, vessels, heart and kidneys either directly or via the release of cytokines leading to hypertension and end organ damage [61]

1.2.4. Ang II Enhances Inflammation

Ang II, the end-product of RAAS, exacerbated and enhanced inflammation and the immune system [61]. Zubcevic et al. discovered that the injection of Ang II in Wistar-Kyoto rats led to increased inflammatory $CD4^+$ T cell accompanied by the increase in IL-17 and activation of the microglia paraventricular nucleus in the hypothalamus [63]. It was also shown that Ang II administration activated the splenic nerve, positively altering the gene expression of pro-inflammatory cytokines from the immune cells in the spleen [64]. Furthermore, Ang II participates in the differentiation of the naïve CD4+ T cells into T helpers (1 and 2) via AT_1R through the activation of NF-κB and STAT1 [65]. Therefore, this activation of NF-κB promotes vascular inflammation and injury, accelerating atherosclerosis and aneurysm [66]. To wrap up,

Ang II facilitates a plethora of inflammatory processes: 1) endorsing the expression of the vascular endothelial growth factor (VEGF), the endothelial adhesive molecule, the adhesion molecules and integrins increasing the vascular permeability, thus causing vessel injury, 2) activating cyclooxygenase 2 which generates ROS and prostaglandins producing a dysfunction to the endothelium, 3) stimulating the release of chemokines and cytokines such as the monocyte chemoattractant promoting the activation and infiltration of inflammatory cells (macrophages, DCs, and T cells) into tissues, 4) provoking the expression of Toll-Like receptor 4 which encourage apoptosis, inflammation and cellular oxidative injury, 5) activating T cells via AT_1R controlling its proliferation and infiltration, the release of more cytokines, the production of ROS, and the NADPH activity [66].

1.2.5. Vascular Remodeling and Immunity

The vessels wall comprises endothelium cells, smooth muscle cells, adipose tissue, fibroblasts that determine the shape, provide protection, and ensure interaction and signaling and detect environmental changes. Those vessels are subjected to remodeling during cellular death, cellular growth, cellular migration, and the degradation of the extracellular matrix formation. This remodeling involves the connections between vasoactive substances, hemodynamic stimuli, and local growth factors, and it happens during hemodynamic changes contributing to vascular and circulatory diseases. Vascular remodeling changes affect the media to lumen ratio of the vessels in several ways first by modifying the wall width when the muscle mass is increased, or in the presence of cellular and non-cellular elements reorganization, second by changing the dimensions of the vessel s lumen and third by causing

microcirculation rarefaction which means that the capillary zone is lost. This vessels remodeling will promote hypertension and vessel injury [67]. In Hypertension, small artery remodeling occurs in two ways either a hypertrophic way where there is an increase in the cross-sectional media area and the media/lumen ratio due to the thickening of the media or in an inward eutrophic way in which the cross-sectional area of the media is intact, an increase in the media/lumen ratio is seen, and the lumen and outer diameters are decreased. Apoptosis is characterized by regulated cell death [68], by inflammation-mediated through the proliferation of immune cells with the release of IL-17A [69], and by chronic vasoconstriction characterized by extracellular matrix remodeling. During hypertension the inflammation and vasoconstriction contribute to vascular remodeling eventually becoming maladaptive leading to cardiovascular injuries [68]. Throughout remodeling, the vessel wall cells start to secrete and release cytokines, ROS encouraging more remodeling of the vessels and surrounding matrix. This remodeling occurs with no pressure increase; however, the resulting resistance of the vessel is a significant factor of hypertension [70]. Nevertheless, it is not fully clear if the remodeling is the cause of vascular damage initially, promoting the infiltration of the inflammatory immune cells further damaging the vessels and organs [61].

1.3. Phosphate

Phosphate in food exists in two types: the *organically bound* phosphate present in protein-rich food such as meat, potatoes, bread, and the *free-inorganic* phosphate added to food and serves as a preservative. The difference between these two forms is that the organic phosphate is not absorbed while the free form is absorbed in the gastrointestinal tract [71]. A daily regular diet contains 0.3% of phosphate while

anything above 0.3% is considered as high phosphate intake [72]. Studies have shown that consuming a high amount of processed and fast food containing free phosphate increased their risk of developing hyperphosphatemia, resulting in kidney disease, vessel calcification, and cardiovascular events. Also, patients diagnosed with chronic kidney disease (CKD) who consume a high amount of food containing free phosphate are at risk of disease augmentation or even death. It has been known that phosphate is absorbed through the intestine, and then most of it is excreted without any problems by the kidney via urine and the rest through the feces. However, according to Ritz et al.[71], it has been discovered that phosphate homeostasis is mediated by phosphate/Fibroblast growth factor 23/klotho endocrine axis. Klotho, a beta-glucuronidase, is a co-receptor for FGF23 [71]. Its absence will stop the effect of FGF23, leading to hyperphosphatemia and elevated active vitamin D. Thus, the presence of FGF23/klotho will increase the phosphate excretion, therefore, delaying the progression of hyperphosphatemia in normal individuals or patients with renal failure. It is necessary to inform the population about the harmful effect of the excess consumption of food with added free phosphate and push the manufacturers to state the exact amount of added phosphate and the total phosphate concentration present in each product by properly labeling them [71].

1.3.1. Phosphate Metabolism and Disturbance

In rocks, phosphorous is found in an oxidized form and highly reactive free element, which can assume many forms being yellow, red, violet, black, or white, while white phosphorous is the most volatile and subjected to a spontaneous explosion. Henning Brand, in 1669 was able to be the first one to isolate phosphate from 50

buckets of distilled human urine while he was searching for the material that can change any metal into gold known as the "philosopher's stone". However, the phosphorous isolation did not fulfill his goal, he had discovered an essential element involved in many life aspects and crucial for survival, where he named it phosphorous or phosphate [73]. In the human body, phosphate is the main component of bone and teeth (85%), and the rest are distributed as 14% in tissue and 1% in the extracellular fluid. It is implicated in many biological processes ranging from being responsible for the mineralization of bones and teeth, to being the backbone of the structure of the nucleotides for RNA, DNA and ATP and in the structure of the cell membrane, to being involved in cell signaling through phosphorylation of enzymes and to being responsible for the acid-base homeostasis especially buffering of the urine [73, 74]. The only source of body phosphate is the diet intake of either the organic or inorganic form of it from the 24-hour food consumption, where the recommended daily allowance should not surpass 700 mg per day and the estimated average requirement is 580 mg per day. However, this amount is usually exceeded by 2 folds in the American diet [75]. Foods that include phosphate are dairy products (eggs, milk), meat, fish, nuts, and food that comprise additives and preservatives. Many studies have shown that adding excessive phosphate to foods can lead to serious health problems affecting the heart, bones, and kidneys since the level of circulating phosphate will exceed the normal level ranging from 2.5 to 4.5 mg/dl. Karp et al. observed in 14 healthy women that the excessive consumption of foods with phosphate additives increased its serum level for 10 to 20h after the meal feasting. In contrast, a study on 3368 participants by Dhingra et al. showed a 55% risk of having cardiovascular disease in the future in people with serum phosphate levels over 3.5 mg/dl compared with those who have a lower phosphate

levels [76]. Phosphate metabolism in the human body is regulated by the three mechanisms: (1) the dietary intake and intestinal absorption, (2) the calcitriol involved in the phosphate uptake from the bones and gut, and (3) through the parathyroid hormone (PTH) responsible for the phosphate resorption from bones and its effects on the proximal tubule and production of calcitriol [74]. Here is a summary of the normal homeostasis of phosphate in the human body. To start with the intestinal absorption regulation, 40% to 80% from the daily 1500 mg phosphate intake is absorbed in the intestine and the rest is excreted through the feces. It is important to mention that the type of diet and phosphate have an impact on its intestinal absorption since the diet that contains high phosphate in the form of additive slat or preservatives is readily absorbed compared to the phosphate present in plants as phytic acid since it is not bioavailable for humans because they need an special enzyme "phytase" to degrade and absorb it. This intestinal absorption can occur in two pathways either (1) through the tight junctions via a passive paracellular way or (2) through the sodium-dependent phosphate co-transporter Npt2b via an active way. The active phosphate transport is characterized by an increase in the expression of Npt2b on the intestinal epithelial cells on the apical side regulated by 1,25-dihydroxyvitamin D known as calcitriol [77]. below [73] is a representation of phosphate homeostasis, in part A during a low serum or dietary phosphate, the parathyroid gland calcium-sensing receptor stimulate the reduction in the production of PTH while co-transporters PiT1/PiT2 in the bones contribute to the decrease in the release of the fibroblast growth factor 23 (FGF23) as shown in Figure 5 by Hernando et al. [73]. The low levels of PTH and FGF23 will therefore act on the kidney and mediate its phosphate reabsorption in the proximal convoluted tubule by increasing the expression of Npt2a (SLC34A1) and Ntp2c (SLC34A3) in addition to

activating and releasing vitamin D (1,25-dihydroxyvitamin D) to help the increase in the dietary phosphate absorption at the level of the intestine via stimulating the expression of Ntp2b (SLC34A2). In contrast, when the phosphate level is high (part B) the same calcium receptors of the parathyroid gland and the co-transporters PiT1/PiT2 of the bones will sense this increase leading to increasing the levels of PTH and FGF23/Klotho released, which in turn decrease the expression of Npt2a (SLC34A1) and Ntp2c (SLC34A3) in the proximal convoluted tubules of the kidney decreasing the reabsorption of phosphate back into the circulation promoting its urinary excretion. Moreover, this reduction of PTH/FGF23 results in a less 1,25-dihydroxyvitamin D release, inhibiting phosphate absorption from the intestine via diminishing expression of Npt2b (SLC34A2) (figure 5). At the cellular level, phosphate balance is mediated by the sodium-dependent phosphate co-transporters SLC20 family, which help in the phosphate uptake into the cells implicated mainly in bone development where any abnormalities in this family will lead to vascular calcification and bone structure abnormalities [73].

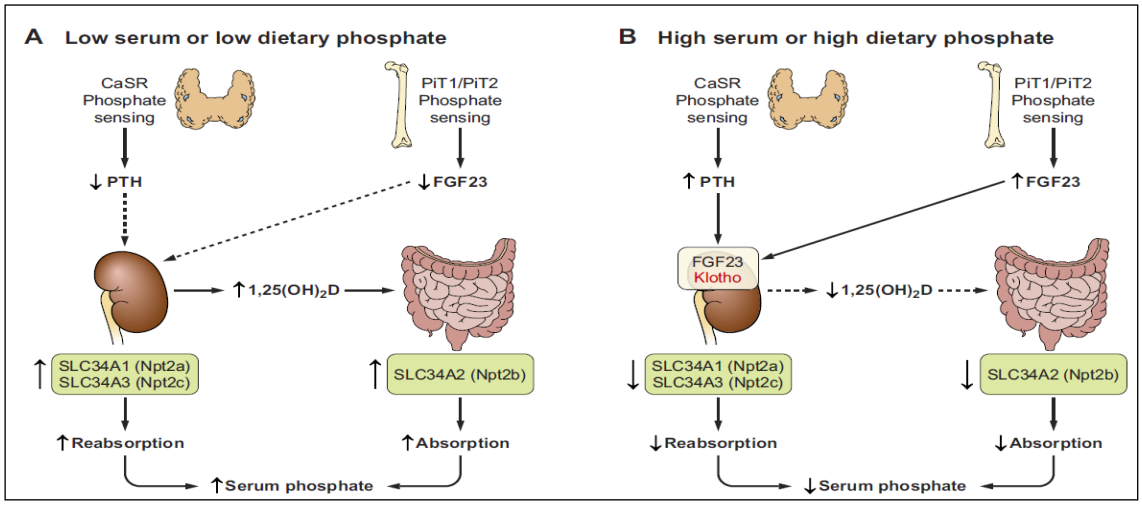

Figure 5. Homeostasis of serum phosphate in the human body. In part A, during a low serum or dietary phosphate, PTH and FGF23 levels will decrease, leading to the increase in the expression of the sodium-dependent phosphate co-transporters SLC34A1 (Npt2a) and SLC34A3 (Ntp2c) in the kidney, while the vitamin D (1,25-

dihydroxyvitamin D) levels increases stimulating the expression of SLC34A2 (Ntp2b) in the intestine. This channels expression increase will lead to an elevated reabsorption and absorption resulting in increased serum phosphate. In part B when the serum and dietary phosphate level are high, PTH and FGF-23 levels increases and vitamin D decreases resulting in a decreased expression of SLC34A1 (Npt2a) and SLC34A3 (Ntp2c) and SLC34A2 (Ntp2b) decreasing the reabsorption and absorption of phosphate promoting the decrease of serum phosphate. [73]

An array of disorders occur, leading to altered phosphate levels. High phosphate levels are usually above 4.5 mg/dl, known as hyperphosphatemia, which is associated with chronic kidney disease (CKD) and cardiovascular diseases since patients suffering from CKD have a diminished kidney function. Therefore, an impaired phosphate excretion resulting in phosphate retention and eventually an increase in the production of FGF-23 and PTH. This increase in FGF-23 will probably be the source of left ventricular atrophy since it stimulates the cardiomyocytes' pathological hypertrophic gene. Also, CKD patients have a deficiency in klotho , which might cause a disturbance in the function of the endothelial cells and increase the calcification of the arterial wall and promote valvular disease [78]. Moreover, a high phosphate intake can cause many metabolic changes that lead to clinical disease apart from CKD such as osteomalacia due to high PTH, osteoporosis due to high FGF-23, ectopic calcification due to low vitamin D, and aging due to low klotho.

On the other hand, a low serum phosphate level (hypo-phosphatemia) can be due to a physiological acquired condition like tumor-induced rickets/osteomalcia where a tumor secretes phosphatonin, causing the inhibition of the phosphate proximal tubule reabsorption resulting in hypophosphatemia eventually impairing the skeletal mineralization contributing to rickets/osteomalacia. Also, hypophosphatemia can be the result of several genetic mutations. First, the phosphate-regulating gene with homologies to endopeptidase on X chromosome mutation (PHEX) also known as X-

linked hypophosphatemia leads to growth retardation and rachitic bone disease. Second, the FGF-23 gene mutation causing a disease knows as autosomal dominant hypophosphatemic rickets characterized by a short stature, bone deformity and phosphate renal wasting. Third, the Npt2a gene mutation contributing to nephrolithiasis and low bone mass. Last the FGF receptor 1 gene mutation causes a disease named Craniofacial dysplasia with hypophosphatemia characterized by craniofacial deformities, depressed nasal bridge, renal phosphate wasting, and short limb dwarfism. All this brings us to a conclusion that phosphate homeostasis is crucial to maintain a normal level of phosphate in the serum at all times to avoid any serious health problem that might accompany it [79].

1.3.2. Phosphate, Hypertension and End-Organ Damage

Several studies and publications have discovered that dietary and serum phosphate affect blood pressure and cause end-organ damage to the kidneys. These studies discovered a negative effect of phosphate on BP, causing its rise and accelerating the kidney disease burden. Bozic et al. conducted a study to test the effect of high serum phosphate on arterial pressure in rats. They fed rats for 4 weeks either a high phosphate diet containing 1.2% P and 0.6% Ca or a moderate phosphate diet containing 0.6% P and 0.6% Ca. In their study, they discovered that after 4 weeks the animals on high phosphate diet showed a significant increase in their BP, PTH, renin and Ang II levels which was reversed after adding a phosphate binder, the lanthanum carbonate, stressing on the fact that phosphate is the one that increased BP and not any other dietary component. This leads them to conclude that a high phosphate diet increases BP, paralleled by an increase in renin through PTH [80]. While Mohammad et

al. [81] discovered similar results in humans after they conducted a prospective study on 20 adults with normal renal function divided randomly into two groups, the first subjected to a high phosphate diet adding to their regular diet 1mmol/kg body weight per day of Na as sodium phosphate. In contrast, the second group was subjected to a low phosphate diet, adding to their regular diet 0.7 mmol/kg body weight per day of Na as NaCl, both for 6 weeks. After those 6 weeks, the group who took a high phosphate diet showed a significant increase in the SBP by 4.1 mmHg and DBP by 3.2 mmHg compared to the group that took a low phosphate diet and increased the heart rate by 4 beats per min. Therefore, high dietary phosphate can increase the BP in humans with normal kidney function, particularly by positively affecting the sympatho-adrenergic activity [81]. Moreover, Zhang et al. [82] executed a cross over study in young, healthy adults separated into 3 groups exposed for 5 days only to a high phosphate diet (2300 mg/day), normal phosphate diet (1500 mg/day), and low phosphate diet (500mg/day). They discovered that the high phosphate diet pointedly raised the SBP compared to the other diets [82]. Those findings were also kind of similar to McClure et al. [83] study on young adults exposed to 1154 mg of phosphate daily from different sources like plants, meat, additives for 6 months. The total phosphorus did not significantly affect BP; however, the added phosphorus displayed a significant rise in SBP and DBP by 1.24 mmHg and 0.83 mmHg, respectively, suggesting that not only the amount but also the type of phosphate can modify the BP [83]. It has also been discovered that high serum phosphate levels can increase the risk of cardiac and kidney diseases in individuals free from any CVD or CKD [84, 85]. According to Park et al. [86], a normal serum phosphate level should always be maintained to avoid health problems in healthy humans. Their study showed that hyperphosphatemia has been correlated with

cardiovascular disease with increased mortality and morbidity. While hypophosphatemia has been correlated with a metabolic syndrome characterized by hyperglycemia, obesity, hypertension, and dyslipidemia, which is also a risk factor for cardiovascular events even in patients with normal kidney function [86]. In contrast, several studies opposed the previous ones stating that phosphate decreases blood pressure. Conferring to Bindels et al. [87], spontaneously hypertensive rats (SHR) suffer from hypophosphatemia compared to normotensive Wistar Kyoto rats (WKY). Their study showed that the administration of a high phosphate diet in drinking water (2% of K2HPO4.KH2PO4) reduced serum phosphate level in SHR yet had no effect on the serum levels of WKY. Also, they discovered that the blood pressure of the SHR taking phosphate diet decreased significantly after 15 weeks of the diet initiation, while a slight but significant decrease in blood pressure was also seen in phosphate supplemented WKY after 19 weeks of diet initiation [87]. Another study including 35 hypertensive men was performed where their BP, plasma phosphate, plasma epinephrine, and heart rate were recorded and evaluated. Kjeldsen et al. declared a negative correlation between blood pressure and serum phosphate and a positive correlation between blood pressure, plasma epinephrine, and heart rate [88]. Kesteloot and Joosens [89] also proved this negative correlation between plasma phosphate and blood pressure in 1988 after measuring, comparing and analyzing the BP and serum phosphorus in 3891 women and 4167 men [89]. A more recent study in 2008 by Elliot et al. [90] recruited in a cross-sectional study 4680 men and women aged between 40 and 59 years old . They recorded BP for eight times during 4 visits and their dietary consumption. Interestingly, the SBP and DBP in individuals with high dietary phosphate consumption (232 mg/1000 kcal) was reduced by 2.3 mmHg and 1.4 mmHg, respectively [90]. Since this

topic is under an emerging controversy, each explanation relies on different dietary phosphate percentages, correlations, and different study designs, which drove us to test the relationship between phosphate, blood pressure, and end-organ damage.

CHAPTER 2

OBJECTIVE OF THE STUDY

In my thesis, I hypothesized that dietary phosphate reduces blood pressure, inflammation and end-organ damage. I hypothesized that high serum phosphate decreased sodium-dependent phosphate co-transporter expression, leading to a decrease in sodium water retention and, therefore, a decrease in BP, but this is related to specific phosphate concentrations and not any random concentrations. Concerning the attenuation of inflammation, a high phosphate diet (1.5%P) might lessen the infiltration of the immune cells in the kidneys and decrease the cytokine levels in the serum. This hypothesis will be investigated by comparing the BP of normotensive and hypertensive mice fed low, control, and high phosphate (P) concentrations (0.15%, 0.3%, and 1.5%) respectively, by assessing the kidneys' and bone marrow's immune cells' infiltrations, and by testing kidney damage. In this study, we focus on three aims in order to efficiently test our hypothesis and prove it. The first aim is to determine the effect of different concentrations of dietary phosphate on the blood pressure of hypertensive mice. The second aim is to study the effect of dietary phosphate on kidney and bone marrow inflammation and immune cells infiltration in hypertensive mice compared to normotensive ones. The third aim is to study the role and effect of dietary phosphate on kidney damage in hypertensive mice.

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1. Animal Model and Approval

In this study, wild-type male C57BL/6 mice were recruited at age 3-months from the Animal Care Facility of the American University of Beirut. They were housed at room temperature on a 12-hour light and dark cycle and had access to water and food (either to Teklad diet or phosphate diet) *ad libitum.* At the end of the experimental protocol, mice were sacrificed by CO_2 inhalation. This study and all the procedures performed were approved by the American University's Animal Care Facility - The Animal Ethics Committee - and conveyed in agreement with the Institutional Animal Care and Use Committee Guidelines for the Ethical Use of Animals for research purposes.

3.2. Study Design

The study protocol is summarized in figure 6 below. In brief, mice were subjected to 1-week blood pressure adaptation to be familiarized with the machine followed by 1-week of baseline BP recording, during which they were fed regular chow *ad libitum*. Hereafter, mice were fed a normal control diet for 1-week in the form of a cookie then habituated to the type and shape of the food. Then, mice were divided into two groups; thirty-five mice were infused with 490 ng/kg/min of Ang II for 28 days using subcutaneous mini-osmotic pumps (Alzet, model 2004) and the rest were considered control/ sham group that received saline infused pumps. Following pump implantation, all the mice were fed a 0.3% phosphate control cookie diet for two weeks.

Then, mice were fed a diet that contains phosphate with different percentages; low 0.15%P, control 0.3%P and high 1.5%Pfor two weeks;. At the end of the protocol, all mice were sacrificed, and the kidneys were harvested, whereas blood and urine were collected for further analysis (figure 6). Throughout the protocol, we used the Visitech for non-invasive tail-cuff BP measurements. In the future, we will use telemetry to assess quantitative BP differences.

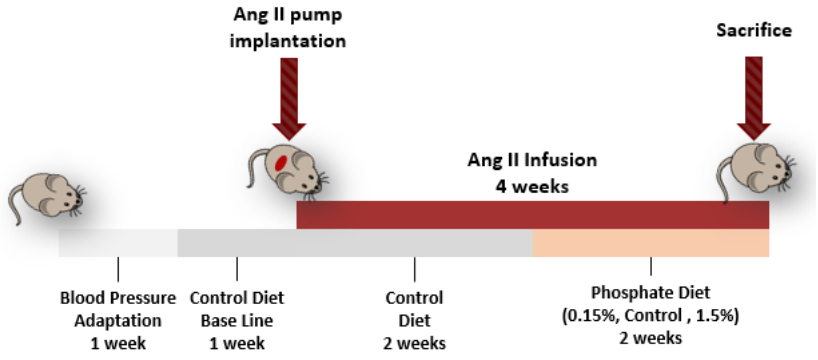

Figure 6. Study design

The composition of each diet given to those mice contains many components. They differ mainly in their potassium phosphate content, sodium chloride, and potassium chloride. Table 2 shows the composition of each diet given to the mice during this study.

Table 2. Diet composition

Ingredients	Normal control group	0.15% P	Control 0.3% P	1.5% P
Casein	200	200	200	200
L-methionine	3	3	3	3
Starch	400	367.7	364.7	340.9
Sucrose	232	232	232	232
Oil	70	70	70	70
Cellulose	50	50	50	50
Min mix	35	35	35	35
Vit mix	10	10	10	10
Potassium Phosphate	0	0	6.5	59.1
Sodium Chloride	0	0	0	0
Potassium Chloride	0	32.3	28.8	0
Total wt g	1000	1000	1000	1000

3.3. Blood Pressure Measurements

BP-2000 Blood Pressure Analysis System Series II from the Visitech is used to measure mice BP weekly via a non-invasive tail-cuff throughout the protocol. BP measurement is mediated by an LED light source that detects the pulse's wave signal. Because the machine is motion-sensitive, the procedure of BP necessitates adaptation sessions for the mice to acclimate mice and give accurate results. Mice are placed on a pre-warmed platform restrained by a magnetic device keeping the tail accessed to insert and slide the entire corresponding tail cuff. In each cycle, the BP unit will start pressurizing to detect a pulse; once detected, it starts pressurizing and depressurizing to give us the systolic and diastolic pressure with the heart rate. These data were exported into excel files to be statistically analyzed. Thirty runs , 3 sets of 10 measurements, are performed for each mouse to obtain significance, where the first ten readings are excluded, while the remaining 20 are averaged and included in this study. Note that the software automatically excludes any failed BP measurement recorded by the system.

3.4. Single-Cell Suspension Preparation

At sacrifice, the kidneys were harvested and processed to isolate single-cell suspension. The gentleMACS C tubes were used (Miltenyi, 130-093-237) combined with the gentleMACS dissociator system (Miltenyi, 130-093-235) to dissociate the kidney cells mechanically. The homogenization step is followed by adding 2mg/ml of collagenase D (ROCHE, 11088866001), 100 µg/ml of DNAse I (ROCHE, 10104159001), and RPMI 1640 medium with 5% FBS. Then, C tubes are placed in a preheated oven to 37°C while being gently rotated for hybridization for 20 min. The homogenate is filtered via a 40-µm cell strainer into a falcon tube, and then subjected to Percoll (Sigma GE17-5445-02) gradient centrifugation. Cells were isolated and washed in cold phosphate-buffered saline (PBS). These isolated cells will be ready for processing and analysis.

3.5. Flow Cytometry Analysis

Renal cells isolated as described previously are washed with cold PBS, then stained with the LIVE/DEAD® Fixable Violet dead cell stain (Invitrogen, L34955) for 30 min at 4 °C in the dark. Samples are washed with PBS, and stained with specific antibodies for 30 min at 4°C in the dark to subject each sample to flow cytometry. The antibodies used are peridinin chlorophyll protein-cyanin-5.5-conjugated anti-CD3 antibody, allophycocyanin-cyanin-7 conjugated anti-CD4 antibody, Brilliant Violet 510 -conjugated anti-CD45 antibody, phycoerythrin-cyanin-7-conjugated anti-CD8a antibody, APC-conjugated anti-CD44 antibody, and PE-conjugated anti-CD62L antibody, and FITC anti-F4/80, and PE-conjugated anti-CD62L antibody. BD FACS Aria™SORP cell sorter machine is used to perform the flow cytometry procedure, and

the flowJo software is used for data analysis. The fluorescence minus one (FMO) control is used for the analysis of the immune cell subset gating. CD4+ and CD8+ T cell subpopulations (the central and effector memory cells) are enumerated within the $CD45^{+}CD3^{+}$ gate. Figure 7 represents the gating strategy followed where the total number of infiltrating cells is calculated by dividing each population total cell number by the amount of the live cells attained during the flow cytometry process leading to the expression of results (the number of cells per tissue). Finally, the number of immune cells analyzed by flow cytometry using the above multiple fluorochrome panel were exported based on the FMO gating strategy. Note, that the control FMO comprises all the fluorochromes in the panel except the one measured.

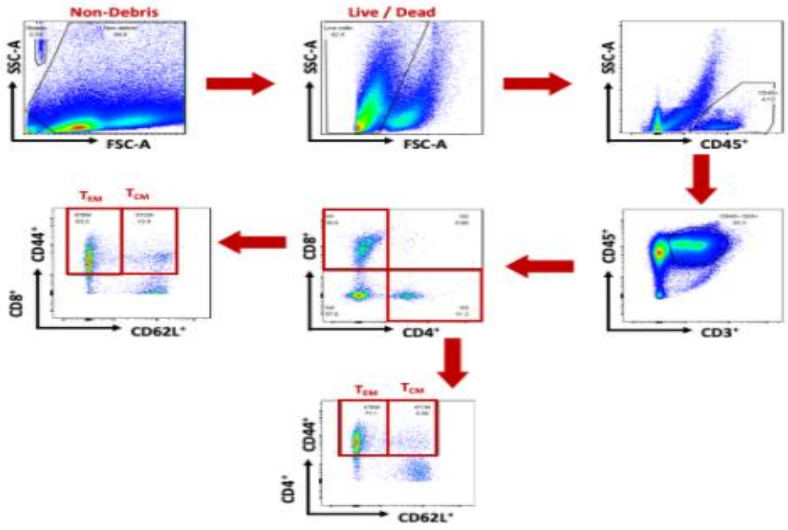

Figure 7. Gating strategy of immune cells. We drilled down for live singlets, then stained for T cells, then differentiated between CD4+ and CD8+ cells, and finally differentiated between Central memory cells and Effector memory cells for each category.

3.6. Measurements of Renal Injury

To assess renal glomerular damage at the end of the study protocol and diet intervention, 24-hour urine samples were collected to measure albumin: creatinine ratio

since albuminuria is a marker for glomerular kidney damage. Therefore, to collect urine samples, at the end of the protocol and before the sacrifice day, each mouse is placed in an individual MMC100 Metabolic Cage, a free-standing unit design made to accommodate one mouse for 24-hours with access to water. The MMC100 Metabolic Cage is drained over a mesh wire where feces are trapped, and uncontaminated urine is collected in a glass flask (figure 8). After 24 hours, urine samples are collected, transferred to Eppendorf tubes, centrifuged at 10k rpm for 10 minutes to remove any particles and debris that might affect our results, and stored at -80°C freezer until albumin testing. Urinary albumin and creatinine levels are determined by using Enzyme-Linked Immunosorbent Assay (ELISA) kits.

Figure 8. MMC100 Metabolic Cage [91]

3.7. ELISA

ELISA was performed following the Albuwell M kit and the Creatinine Companion kit instructions. Enzyme-Linked Immunosorbent Assay (ELISA) detects specific antigens using a particular target antibody to form antibody-antigen interactions. ELISA is performed in a 96 multi-well plate, which gives a solid surface for the immobilization of the antigen to be detected. This immobilization is important in

facilitating the separation process of the specific antigens from the residues present per sample. In brief, the antigen is immobilized directly or by capturing antibodies to the wells' solid surface. The specific antibody used for detection is then conjugated with a molecule acquiescent for being detected like an enzyme or a fluorophore. It is then added to the wells that contain the specific antigen where it binds to it and form the antigen-antibody interaction (figure 9). All this makes ELISA an easy assay used to detect many antigens performed on samples simultaneously. To detect the albumin present in the urine, we centrifuged the sample at 1000 gms to eliminate the debris and dead cells.

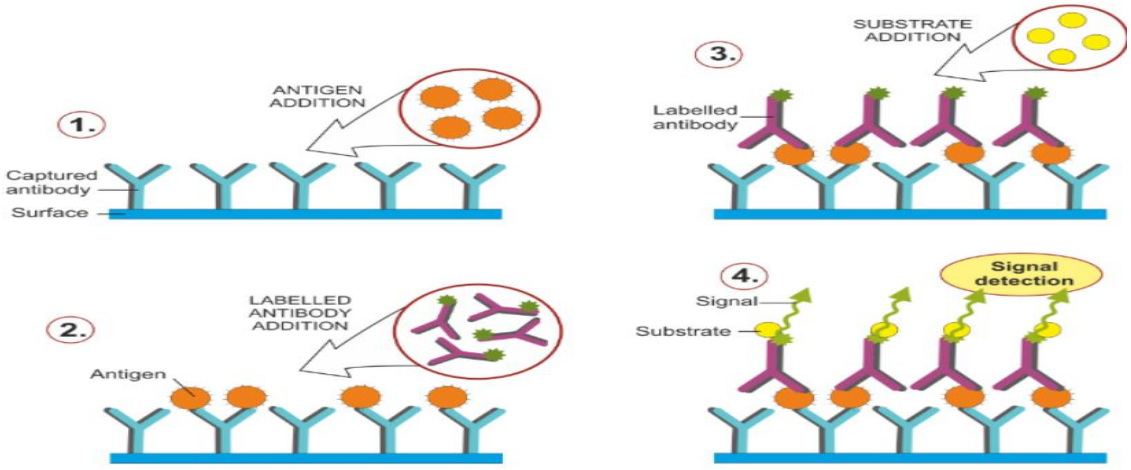

Figure 9. Enzyme-Linked Immunosorbent Assay [92]

3.8. Staining for Kidney Analysis

After fixing the kidney in formalin, it undergoes histochemical treatment to have ready-to-be-stained kidney slides. These slides will be stained by Hematoxylin and Eosin (H&E), by Dihydroethidium (DHE), by Masson's trichrome blue, and by Periodic-acid Schiff (PAS).

H&E stain: Hematoxylin stains mainly the DNA in the cellular matter by binding negatively to the phosphate groups present in the backbones of the DNA, producing a blue color in neutral to basic conditions. In contrast, the eosin binds to the positively charged proteins making a pink color. In the kidney, lymphocyte infiltration is stained by the hematoxylin blue stain, while the eosin stain the hyalinization (tissue degeneration) of the Bowman's capsule. Therefore, a normal kidney H&E stain will make the Bowman's capsule appear as an open space with no inflammation [93]. A light microscope is used to visualize the slides.

DHE stain: this specific stain is used to detect ROS (superoxide) in the tissues like the kidneys since it uses a fluorescent probe to assess its presence. The cells ' cytosol will be stained blue until oxidized in the kidney while the cells 'nucleus appears bright fluorescent red. The DHE kit d11347 is used to stain kidney samples from mice subjected to a different percentage of phosphate diet, where a concentration of 20 um of DHE is used to stain each kidney section A fluorescent microscope is used to visualize the slides.

Masson's Trichrome Blue stain (MTS): this stain is used to detect fibrosis developed in the tissues like the kidney. Masson's trichrome blue stains all collagen showing the degree of fibrosis present in blue color. Therefore, this strain is used to assess the degree of fibrosis present in the kidneys of the mice fed different percentages of phosphate diet. A light microscope is used to visualize the slides.

PAS stain: is mainly used to highlight or visualize the basement membrane of kidneys glomerulus and tubules to show if any damage has affected these membranes or any tubular atrophy present. It stains the polysaccharides present in the membranes in a dark red to purple color. A light microscope is used to visualize the slides.

The slide images are quantified using ImageJ software. To quantify the slide images stained with DHE we followed the following steps: 1) set the scale, 2) change the image preference to RGB stack making it a black and white image, 3) adjust the threshold accordingly and select the black background and 4) adjust the measurement preferences to measure the area and area fraction%. In addition, to quantify the slide images stained with MTS we followed the following steps: 1) set the scale, 2) change the image preference to RGB stack making it a black and white image, 3) adjust the threshold accordingly and remove the black background and 4) adjust the measurement preferences to measure the area and area fraction%. Finally, to quantify the slide images stained with PAS we followed the following steps: 1) set the scale, 2) precisely draw the borders of the full glomerulus then measure and record the area, 3) draw precisely the borders of the glomerulus without the white retracted part then measure and record the area and 4) measure the glomerular retraction ratio by dividing the area of the retracted glomerulus (3) over the area of the full glomerulus (2).

3.9. Statistical Analysis

All the data obtained in this thesis are presented as the mean ±standard error of the mean. The statistical analysis was performed using GraphPad Prism. For multiple comparisons in experiments involving 2x2 design 2-way ANOVA was used. While for single comparison one-way ANOVA was adapted.

CHAPTER 4

RESULTS

After conducting all the experiments listed above, data were collected, analyzed, and organized.

4.1. Phosphate effect on Blood Pressure

To determine the effect of dietary phosphate on the blood pressure, sham and Ang II infused mice were fed low, control and high dietary phosphate (0.15%, control (0.3%), 1.5% P) respectively for two weeks. The SBP (121.89 mmHg ± 4.044) and DBP (55.53 mmHg ± 2.489) of sham mice fed the control diet (0.3% P) at baseline remained slightly the same throughout the study compared to the end of protocol (119.87 mmHg ± 4.561 ; 59.66 mmHg ± 4.150) respectively. However, the SBP of the Ang II-infused mice fed the control diet (0.3% P) was reduced from 154.4 mmHg ± 4.431 at day 14 to 148.4 mmHg ± 3.823 at day 28 by 6.1 mmHg ± 0.608 . Similarly, the DBP was also reduced from 91.86 mmHg ± 4.573 at day 14 to 83.11 ± 4.198 mmHg at day 28 by 8.75 mmHg ± 0.375 . Wild-type mice blood pressure infused with Ang-II was significantly ($P<0.0001$) high compared to the sham mice during the period where they

are fed control diet (0.3% P) from day 20 to day 28 (figure 10).

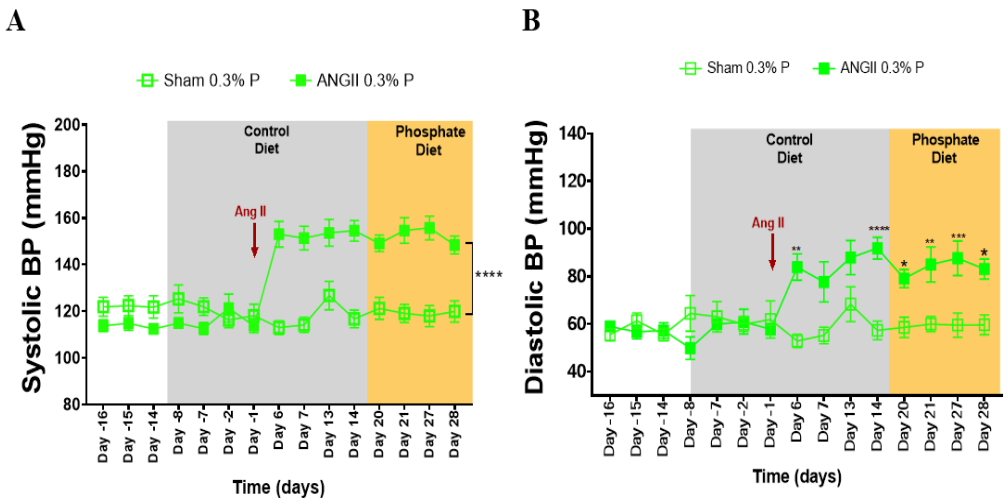

Figure 10. Mouse BP recordings in response to control (0.3%P) dietary phosphate intake in hypertensive mice. Tail cuff (A) SBP and (B) DBP means in response to 4 weeks angiotensin II infusion (490 ng/kg/min). Data were analyzed by 2-way ANOVA with repeated measurements, n=12 and 13 in each group. BP indicates blood pressure. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Similarly, the blood pressure of the sham mice fed the low-phosphate diet (0.15%P) continued to be the same with slight or no fluctuation all along the study protocol being 122.3 mmHg ± 4.601/66.49 mmHg ± 5.467 at baseline and 120 mmHg ± 5.120/63.76 ± 5.474 mmHg at the end of the protocol. In parallel, the blood pressure of the Ang II-infused mice fed normal diet for two weeks (161.7 mmHg ± 4.871/87.25 ± 9.495 mmHg) did not change significantly following the administration of 0.15%P phosphate for another two weeks (157.8 mmHg ± 4.432/77.68 mmHg ± 9.025). Nevertheless, there was a high significance (P<0.0001) comparing the SBP of the sham (120 mmHg ± 5.120) and Ang II-infused mice (157.8 mmHg ± 4.432) , suggesting that the SBP of the latter was not altered following the introduction of 0.15% phosphate in diet (figure 11).

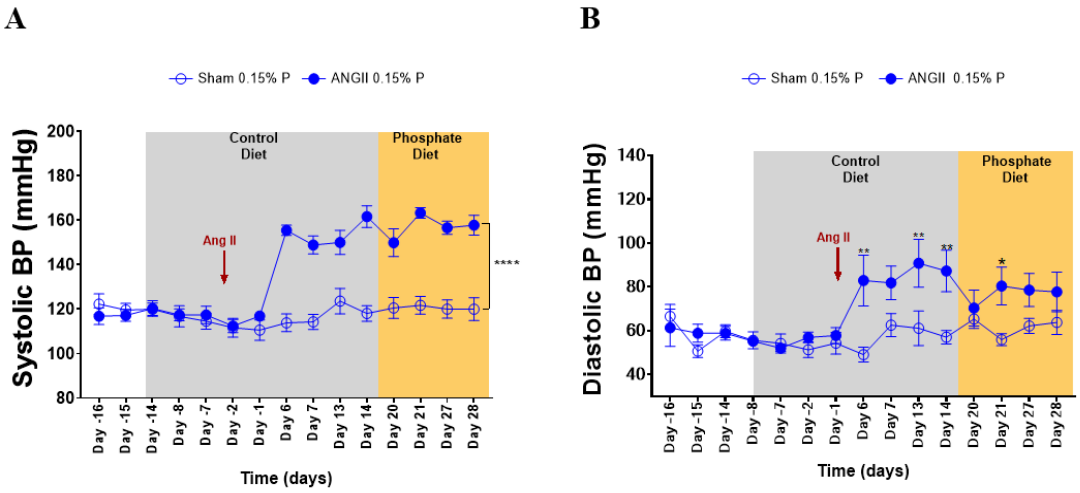

Figure 11. Mouse BP recordings in response to low (0.15%P) dietary phosphate intake in hypertensive mice. Tail cuff (A) SBP and (B) DBP means in response to 4 weeks angiotensin II infusion (490 ng/kg/min). Data were analyzed by 2-way ANOVA with repeated measurements, n=12 and 13 in each group. BP indicates blood pressure. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Finally, sham mice fed a high-phosphate diet (1.5% P) were normotensive with a BP ratio 120 mmHg ± 1.30/58.1 mmHg ± 0.876, respectively. However, Ang II-infused mice were hypertensive at day 14 (159.978 mmHg ± 5.091) as reported in the literature yet upon high phosphate diet (1.5%P) feeding, mice BP was significantly reduced (135.222 mmHg ± 3.076). As seen in figure 12, there was no significant change between the normotensive and hypertensive mice of the high diet group at the end of the protocol (day 28), unlike the sham group. We discovered that the SBP of the hypertensive 1.5%P mice significantly reduced by 24.8 mmHg from 160.0 mmHg at day 14 to 135.2 mmHg at day 28. Likewise, its DBP significantly reduced by 19.32 mmHg from 86.84 mmHg on day 14 to 67.52 mmHg on day 28 (figure 12).

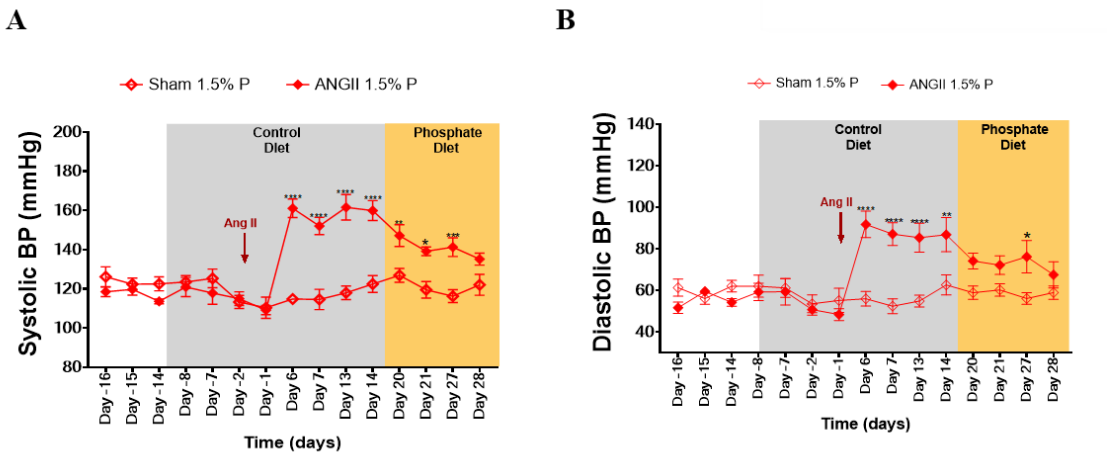

Figure 12. Mouse BP recordings in response to high (1.5%P) dietary phosphate intake in hypertensive mice. Tail cuff (A) SBP and (B) DBP means in response to 4 weeks angiotensin II infusion (490 ng/kg/min). Data were analyzed by 2-way ANOVA with repeated measurements, n=12 and 13 in each group. BP indicates blood pressure. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.2. Phosphate reduces immune cells infiltration and inflammation in the kidney

To determine if dietary phosphate affects the infiltration of immune cells in the kidney in response to hypertensive stimuli, we performed flow cytometry analysis. We focused on CD4+ T and CD8+ T cells that are altered in hypertension. Our results show that the extent of the inflammation and immune cell infiltration in renal cells was augmented in the hypertensive Ang-II infused mice compared to the sham mice fed all % of dietary P. First, the infiltration of lymphocytes (CD45+ cells) , specifically total leukocytes (CD3+ T cells) in the kidney of the Ang II-infused mice of all % of dietary P was reduced compared to the sham group, especially the high phosphate diet (figure 13). Total CD3+ Memory T cells levels, especially that of the effector memory were reduced in the all-hypertensive mice compared to the normotensive mice, the most significant (P<0.05) being those of the high phosphate diet (figure14).

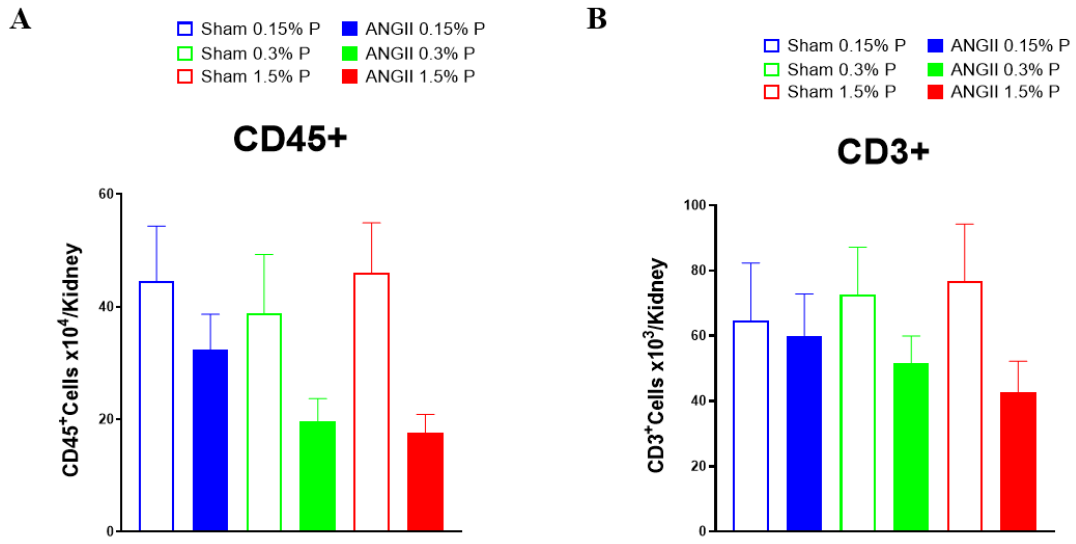

Figure 13. Effect of phosphate on CD45+ and CD3+ immune cell accumulation in the kidney. Summary data from sham mice and 4 weeks Ang II-infused mice (490ng/kg/min) of CD45+ (A) and CD3+ (B) in kidney. N= 6 to 11 per group. Data were analyzed using one-way ANOVA.

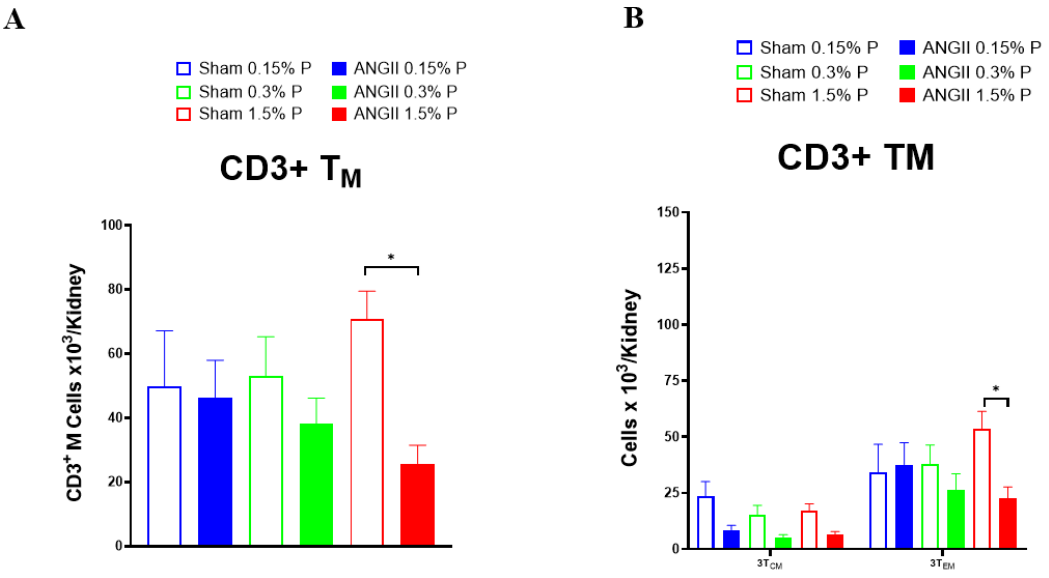

Figure 14. Effect of phosphate on CD3+ immune cell accumulation in the kidney. Summary data from sham mice and 4 weeks Ang II-infused mice (490ng/kg/min) of total memory CD3+ cells (A) and of central and effector memory CD3+ (B) in kidney. N= 6 to 11 per group. Data were analyzed using two-way ANOVA. *P<0.05.

Additionally, CD4+ T cells present in the kidney of the 0.15%P Ang II-infused mice were significantly (P<0.05) less accumulated than those present in the sham

0.15%P mice. The accumulation of CD4+ immune cells in the kidneys of the Ang II-infused mice fed the control (0.3%P) and the high phosphate (1.5%P) diet tends to be also significantly less than the levels present in the kidneys of the normotensive mice (figure 15.A). Concerning the memory of the T helper cells, an exceptionally small number of central memory cells ($4T_{CM}$) were detected in the kidneys of all hypertensive mice compared to the normotensive ones, while the presence of CD4+ effector memory cells ($4T_{EM}$) was exacerbated in the normotensive mice of all % of dietary P groups. We detected a significant ($P<0.01$) decrease of CD4+ effector memory cells in the kidney of the Ang II-infused mice fed the control and high dietary phosphate as seen in figure 15.B.

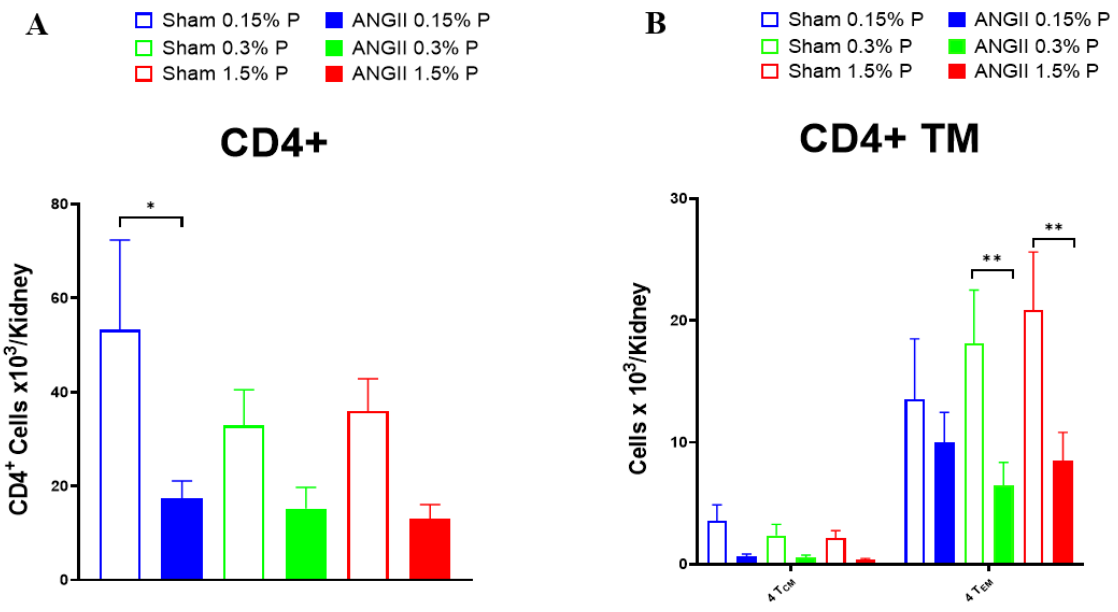

Figure 15. Effect of phosphate on CD4+ immune cell accumulation in the kidney. Summary data from sham mice and 4 weeks Ang II-infused mice (490ng/kg/min) of CD4+ (A) and memory CD4+ (B) in kidney. N= 6 to 11 per group. Data were analyzed using one-way ANOVA for CD4+ and two-way ANOVA for the memory CD4+. *P<0.05, **P<0.01.

Subsequently, CD8+ T cells present in kidneys of the 1.5%P Ang II-infused mice were significantly reduced compared to those present in the sham mice fed 1.5% P

diet. while in the kidneys of the hypertensive mice fed the control and the low phosphate diet the levels of CD8+ are also less than the levels present in the kidneys of the normotensive mice (figure 16.A). Concerning the memory of the cytotoxic T cells, a sparse number of central memory cells ($8T_{CM}$) was detected in the kidneys of all hypertensive mice compared to the normotensive ones, while the presence of CD8+ effector memory T cells ($8T_{EM}$) was intensified in the normotensive mice of all dietary phosphate groups. We perceived a significant ($P<0.05$) reduction in the CD8+ effector memory T cells in the Ang II-infused mice kidney of the high phosphate group (1.5%P) compared to the 1.5%P normotensive mice kidney, while the reduction in the other dietary phosphate groups was not as significant as the high phosphate group, seen in figure 16.B.

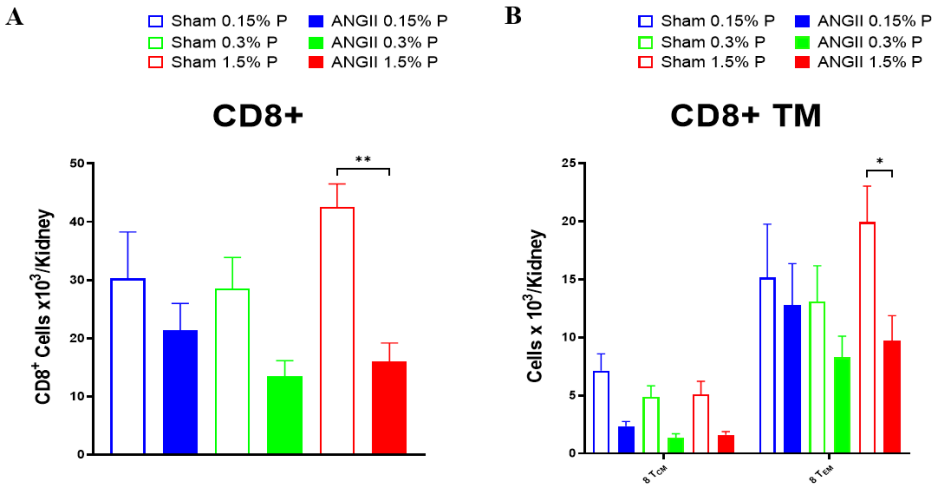

Figure 16. Effect of phosphate on CD8+ immune cell accumulation in the kidney. Summary data from sham mice and 4 weeks Ang II-infused mice (490ng/kg/min) of CD8+ (A) and memory CD8+ (B) in kidney. N= 6 to 11 per group. Data were analyzed using one-way ANOVA for CD8+ and two-way ANOVA for the memory CD8+. *P<0.05, **P<0.01.

4.3. Phosphate effect on renal injury

4.3.1. Albuminuria

To address this study's third aim and determine whether dietary phosphorus intake can cause renal injury and albuminuria in hypertension or kidney disease, sham and hypertensive mice were fed a low, control, or high phosphate diet. High dietary phosphorus intake for two weeks in hypertensive mice caused significant albuminuria compared to the normotensive mice, as seen in figure 17 . The albumin-to-creatinine ratio (ACR) of 1.5%P Ang II-infused mice (2.05 ug/mg ± 0.164) was higher by 0.87 ug/mg ± 0.04 compared to the 1.5%P sham mice (1.18 ug/mg ± 0.204). Correspondingly, hypertensive mice fed a high phosphate diet had significantly higher albuminuria than hypertensive and normotensive mice fed the control phosphate diet and hypertensive mice fed the low phosphate diet. There was also significantly more elevated albuminuria in the control hypertensive mice compared to the control sham mice. In contrast, no significant elevation was detected between the sham and Ang II mice fed the low phosphate diet (figure 17).

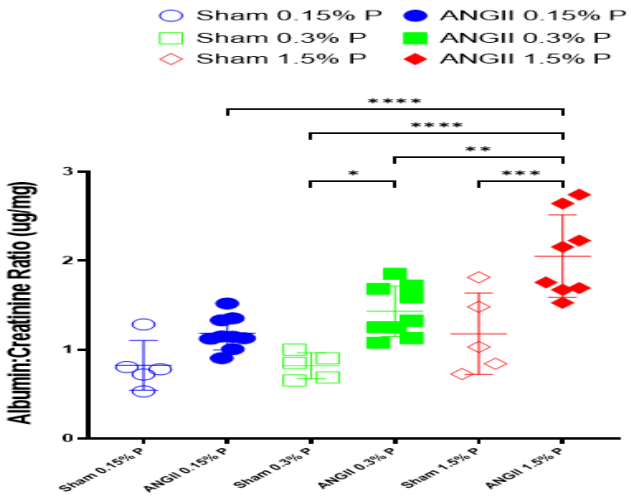

Figure 17. Urinary albumin to creatinine ratio (ug/mg) collected over 24-h from sham mice and 4 weeks of Ang II-infused mice (490 ng/kg/min). Data are expressed as mean

± SEM, n= 5-9 per group. P value analyzed by one-way ANOVA; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.2. Fibrosis

Moreover, Masson's Trichrome Blue stain performed on kidney sections of the sham mice and Ang II-infused mice for all % of dietary phosphate groups (0.15%, 0.3% and 1.5%) was to detect the development of fibrosis; collagen stained blue (figure 17.A,B,C). The fibrosis of the hypertensive control group (0.64 % ±0.034) was slightly higher than that of the normotensive control group (0.53 % ±0.041), indicating Ang II's effect on the kidneys. Likewise, kidney fibrosis of the hypertensive high phosphate group (0.61 ±0.058 %) was significantly higher than that of the normotensive control group (0.31 ±0.034 %). However, 1.5%P Ang II-infused mice and control (0.3%P) Ang II-infused mice kidney fibrosis were approximately the same with minimal or no change suggesting that the increased fibrosis seen in the kidneys of the Ang II-infused mice fed the high phosphate diet is also the effect of Ang II and not that of phosphate (figure 17.D).

In addition, staining the kidney sections with PAS was used to highlight and visualize the basement membrane of kidneys 'glomerulus and tubules to show if any damage has affected these membranes changing the glomerular size or any tubular atrophy present. As seen in figure 17. A,B,C, the polysaccharides present in the membranes are stained in a dark red to purple color. After measuring the size of each glomerulus present in each field, we found out that the glomerular size did not significantly change within the same diet and was also similar between all dietary phosphate groups combined. As we can see in figure 17.E, the glomerular size of sham 0.15%P mice and 0.15%P Ang II-infused mice were 0.74 ±0.016 um^2 and 0.71 ±0.013

um^2, respectively. Those of sham control mice and Ang II-infused mice were close, being 0.70 ±0.0096 um^2 and 0.71 ±0.0064 um^2. Also, those of sham 1.5%P mice and 1.5%P Ang II-infused mice were 0.73 ±0.011 um^2 and 0.71 ±0.0084 um^2, respectively.

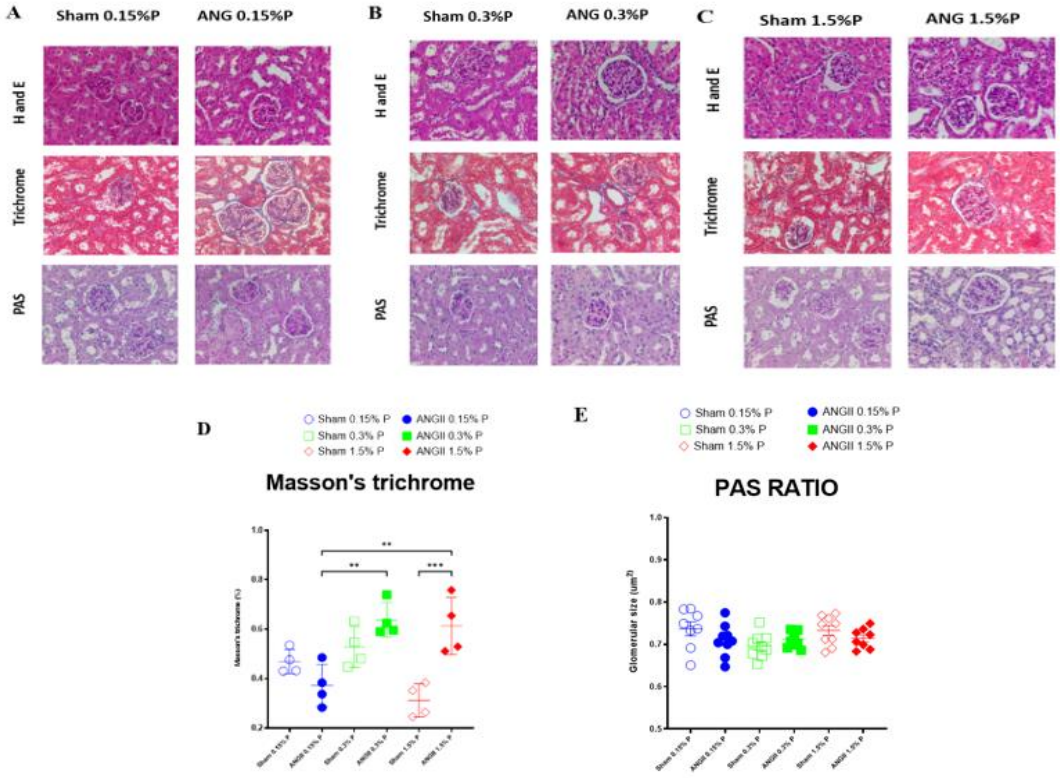

Figure 18.Parameters of renal injury in response to dietary phosphate administered to sham mice and 4 weeks Ang II-infused mice. A,B and C show kidney slides stained by Hematoxylin and Eosin (H&E)for visualization, Masson's Trichrome blue for fibrosis and Periodic acid Schiff (PAS) for glomerular size. D and E show the data expressed in mean ± SEM; n= 8 to 12 per group. P-value analyzed by one-way ANOVA; *P<0.05, **P<0.01, ***P<0.001.

4.3.3. Reactive Oxygen Species (Superoxide)

Finally, to detect the presence of superoxide formation, we stained the kidney sections with dihydroethidium stain (DHE), visualized them using a fluorescent microscope, and quantified them in the ImageJ software. In the kidneys of the mice subjected to low dietary phosphate, the level of superoxide present in the sham was 0.020 ±0.0014 % compared to the Ang II 0.020 ±0.0016 %, which is low compared to

the control (0.3%P) and high (1.5%P)dietary phosphate groups. Hence, we detected a significant increase in the levels of superoxide present in the kidneys between the control (0.3%P) sham mice (0.023 ±0.0013 %) and the control (0.3%P) Ang II-infused mice (0.050 ±0.0067 %). Similarly, the superoxide levels in the Ang II 1.5%P being 0.055 ±0.0070 % were significantly higher than the sham 1.5%P being 0.025 ±0.0016 %. However, let's compare the Ang II groups of the control and high phosphate diet. We discover that they are quite similar with no significant change proving that the presence of ROS is due to the Ang II effect and not due to the addition of a phosphate (figure 19 and 20).

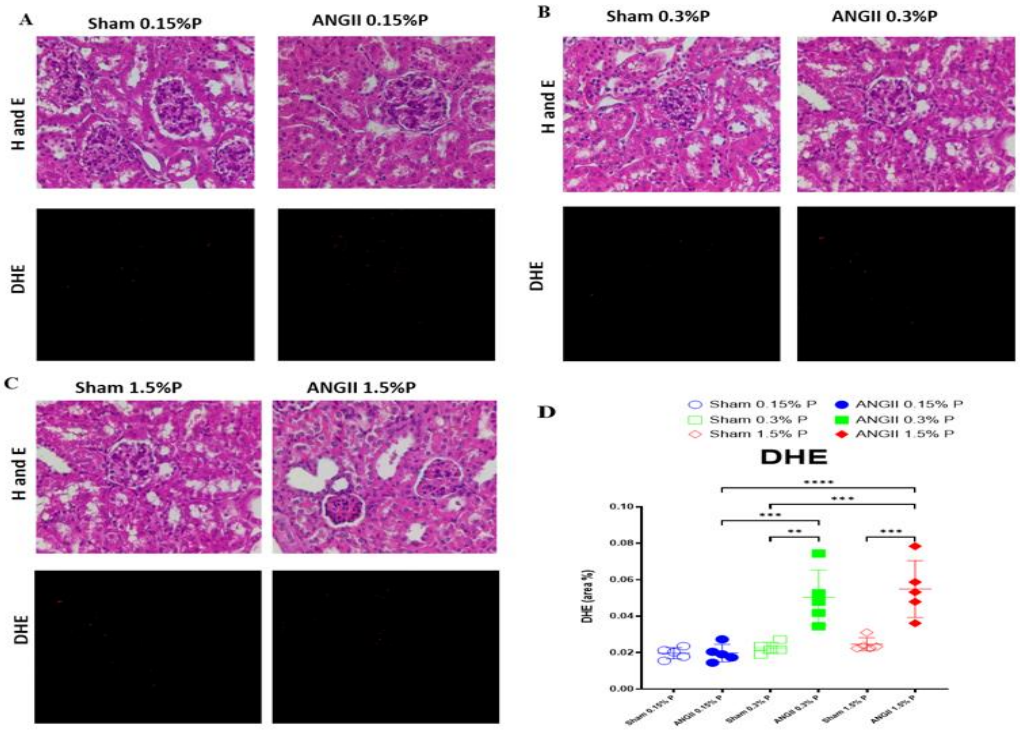

Figure 19. Parameters of renal injury in response to dietary phosphate administered to sham mice and 4 weeks Ang II-infused mice. A,B and C show kidney slides stained by Hematoxylin and Eosin (H&E) for visualization, and Dihydroethidium (DHE) for superoxide detection. D shows the data expressed in mean ± SEM; n= 8 to 12 per group. P value analyzed by one-way ANOVA; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

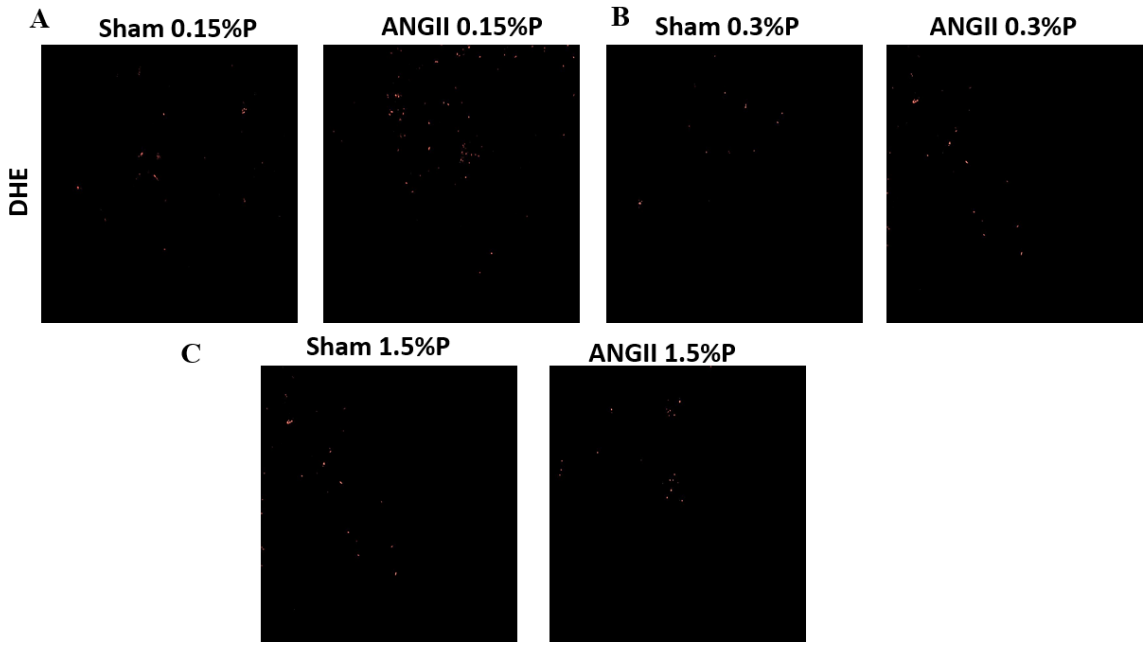

Figure 20. Parameters of renal injury in response to dietary phosphate administered to sham mice and 4 weeks Ang II-infused mice. A,B and C show kidney slides stained by Dihydroethidium (DHE) for superoxide detection; n= 8 to 12 per group.

CHAPTER 5

DISCUSSION

Evidence suggests that dietary phosphate intake is a modifiable risk factor for kidney disease, CVD, and organ damage if administered excessively and uncontrolled [76, 78]. In contrast, it has been shown that a moderate amount of phosphate might be beneficial for the human body and organs [87]. In our study, we focused on three percentages of phosphate diet: low (0.15%), control (0.3%), and high (1.5%) to determine whether phosphorus intake could have adverse or any beneficial effect on blood pressure, inflammation, and end-organ damage. Animal studies demonstrate a direct relationship between phosphorus load in nephron and renal glomerular injury.

First of all, the blood pressure results obtained at the end of this study demonstrate that the administration of a high phosphate diet over a period of 2 weeks to hypertensive mice was able to restore the levels of the blood pressure to normal levels similar to that of the normotensive mice of the same group, unlike, the other two dietary phosphate groups the low 0.15%P and the control 0.3%P, where the BP of the hypertensive mice remained high compared to the normotensive mice of the same group and to the hypertensive mice of the high phosphate group, likewise in previous studies [87, 88, 90]. Those findings can be interpreted by shedding light on the mechanism of how dietary phosphate is metabolized in the body. There are three ways of phosphate elimination, but the most relevant one to our study is through the parathyroid hormone (PTH) and FGF23 responsible for phosphate resorption from bones and its effects on the proximal tubule and production of calcitriol. Therefore, we hypothesize that the reduction in BP following the administration of a high phosphate diet in the

hypertensive mice might be due to the decreased expression of the sodium-dependent phosphate cotransport SLC34A1 and SLC34A3 in the kidneys due to high level of serum phosphate. This decrease will negatively affect the reabsorption of phosphate coupled with sodium through those cotransporters, leading to their elimination in urine and, therefore, decreasing blood pressure [73, 94].

Second, as previously discussed, researchers have shown that the immune cell activation and inflammation in hypertension are evolving, finding that the CD8+ T cells, CD4+ T cells, and B cells produce factors such as Il-17A, IFN-γ, TNF-α promoting hypertension, whereas Tregs inhibit hypertension [95]. It was shown that increased infiltration of human T lymphocytes (CD3+ and CD4+) and leukocytes (CD45+) in the kidney, thoracic lymph nodes, and thoracic aorta of a humanized mouse model treated with Ang II infusion. Also, they observed a high level of IL-17A produced by CD4+ cells and INF-γ produced by CD4+ and CD8+ [51]. Those CD8+ T cells were oligoclonal cells invading the kidney after hypertension and causing sodium and water retention and vascular rarefaction [50]. However, in our study, the flow cytometry data showed a slight decrease in the immune cells 'infiltration, especially CD4+ and CD8+ T cells in the kidneys of the hypertensive control (0.3%P) mice, and a more exaggerated and significant reduction in the hypertensive 1.5%P mice (figures 15 and 16). This data is important since it further asserts that the decrease in BP of the mice fed a high-phosphate diet protects the kidney from immune cell infiltration and inflammation that normally occur during hypertension, as reported in the literature. The precise mechanism of how phosphate decreases kidney inflammation is still unknown. However, the protection of the kidneys from infiltration might also be due to the increased serum phosphate that leads to the reduced expression of the sodium-

dependent phosphate cotransport SLC34A1 and SLC34A3 in the kidneys. This will negatively affect the reabsorption of phosphate coupled with sodium through those cotransporters, leading to their elimination in urine [73, 94], decreasing blood pressure and subsequently attenuating kidney inflammation.

Finally, to assess glomerular kidney damage, 24-h urine was collected to test ACR in urine, and kidney slides were stained with Masson's Trichrome stain to show fibrosis, with PAS to show the change in glomerular size, and with DHE to test the presence of superoxide. The ACR results of the 1.5%P Ang II-infused mice were exaggerated compared to the other 0.15%P and control (0.3%P) Ang II-infused mice groups despite the decrease in BP. It exhibited the most kidney damage since the ACR ratio is higher than that of the control (0.3%P) Ang II-infused mice, meaning that the damage might not be the effect of Ang II alone but the combination of Ang II and high dietary phosphate. Those results correlate with previous studies that discovered that high serum phosphate levels increases [84, 85] kidney damage of the control (0.3%P) diet, especially in the Ang II-infused mice since they had an ACR higher in the hypertensive mice than the normotensive mice is due to the effect of Ang II. Those results can be interpreted by relating the effect of Ang II to renal injury. According to Long et al., Ang II can damage the kidneys in several ways by promoting glomerular and systemic hypertension, by causing ischemia secondary to vessels constrictions, by prompting the activation of renal fibroblasts leading to inflammation, and by resulting in renal tubules injury due to an-induced proteinuria [96] where high levels of albumin can detect this damage. In this case, the damage to the kidneys might have been caused by Ang II before the administration of a high phosphate diet to the 1.5%P Ang II-infused mice because phosphate alone does not significantly affect the ACR according

to Chang and colleagues [97]. Therefore, we think that the exaggerated elevation in the ACR of the 1.5%P Ang II-infused mice compared to the control (0.3%P) Ang II-infused mice may be due to a partially impaired ability to eliminate the high dietary phosphate fed to the mice due to Ang II renal damage. However, the significant decrease in BP in the 1.5%P Ang II-infused mice group can result from several controlling mechanisms such as the kidney effect mentioned above, the central nervous system effect, and the vascular effect [98]. Hence, we can state that the high phosphate diet did not cause glomerular kidney damage because the imaging of kidney sections of 1.5%P Ang II-infused mice compared to control (0.3%P) Ang II-infused mice did not show exaggerated fibrosis, nor glomerular retraction, nor ROS (superoxide) formation as seen in figures 18 and 19. On the contrary, the levels of fibrosis, retraction, and ROS formation in the kidneys of the normotensive mice of each diet group (0.15%, control (0.3%) and 1.5%) were quite like each other and the levels in the kidneys of the Ang II-infused mice of each diet group (0.15%, control (0.3%) and 1.5%) were close to each other indicating that the fibrosis and damage present are the resultant of the Ang II infusion.

The question remains on why the kidneys of Ang II-infused mice fed the low phosphate diet (0.15%P) did not exhibit any fibrosis or any ROS formation? We hypothesized according to previous findings and studies, that the absence of fibrosis and ROS in the kidneys of Ang II-infused mice fed the low phosphate diet (0.15%P) might be due to the decreased levels of FGF-23 [99, 100]. It has been shown that high levels of FGF23 can activate a series of signaling cascade in renal fibroblast promoting fibrosis and ROS release. In brief, FGF23 activates FGFR4 (receptor) leading to the influx of calcium inside the cells increasing its concentration. This increase will

stimulate mitochondrial ROS formation and release, activating therefore TGF-beta1. TGF-Beta1 will lead to another signaling cascade in the same renal fibroblast cell leading to fibrosis formation in the kidneys [99, 100]. However, in our case as we previously discussed, low serum or dietary phosphate will lead to a decrease in the release of FGF23 from the bones (osteoblasts and osteocytes) [73] that might confirm why the levels of fibrosis and ROS are low in the sham and Ang II-infused mice fed the 0.15%P diet. In addition, the results showed that Ang II effect on kidney fibrosis and ROS formation was blunted compared to its effect on the kidneys of the control and high phosphate diet. We suggest that it might be related to the RAAS pathway, since the low levels of FGF23 won't be able to suppress the effect of ACE2 and increasing the Ang II concentration but on the contrary ACE2 will be activated leading to the conversion of Ang II into Angiotensin (1-9) or Angiotensin (1-7) causing ant-inflammation, anti-fibrosis, anti-AngII signaling, vasorelaxation and cardio protection [21, 101]. The question remains on why after all this the BP of the Ang II-infused mice on low phosphate diet remained high, we go back to the phosphate homeostasis cascade. Low serum or dietary phosphate will decrease the levels of PTH, and FGF23 levels, leading to the increase in the expression of the sodium-dependent phosphate co-transporters SLC34A1 (Npt2a) and SLC34A3 (Ntp2c) in the kidney allowing the reabsorption of filtered phosphate by importing 1 molecule of $H_2P0_4^-$ with 3 Na+, and 1 molecule of $H_2P0_4^-$ with 2Na+ molecules, respectively, while the levels of vitamin D (1,25-dihydroxyvitamin D) increases stimulating the expression of SLC34A2 (Ntp2b) which imports 1 $H_2P0_4^-$ with 3 Na+ inside in the intestine. This channels expression increase will lead to an elevated reabsorption and absorption of phosphate and sodium resulting in an increase in BP [73].

To sum up, we were able to achieve all the aims of this study. First, we found that the high dietary phosphate reduces the blood pressure of hypertensive mice. Second, we discovered that high dietary phosphate decreases the inflammation and immune cells infiltration in the kidneys of the hypertensive mice compared to normotensive ones. Third, we suggest that high dietary phosphate might not cause extensive damage to kidneys of the hypertensive mice, but the damage present was due to Ang II infusion over 4 weeks. Figure 21 displays our working model that summarizes all the work and results we have done during our thesis project, where we found that the administration of high phosphate diet to Ang-II infused mice leads to a decrease in the blood pressure, renal inflammation, and glomerular damage.

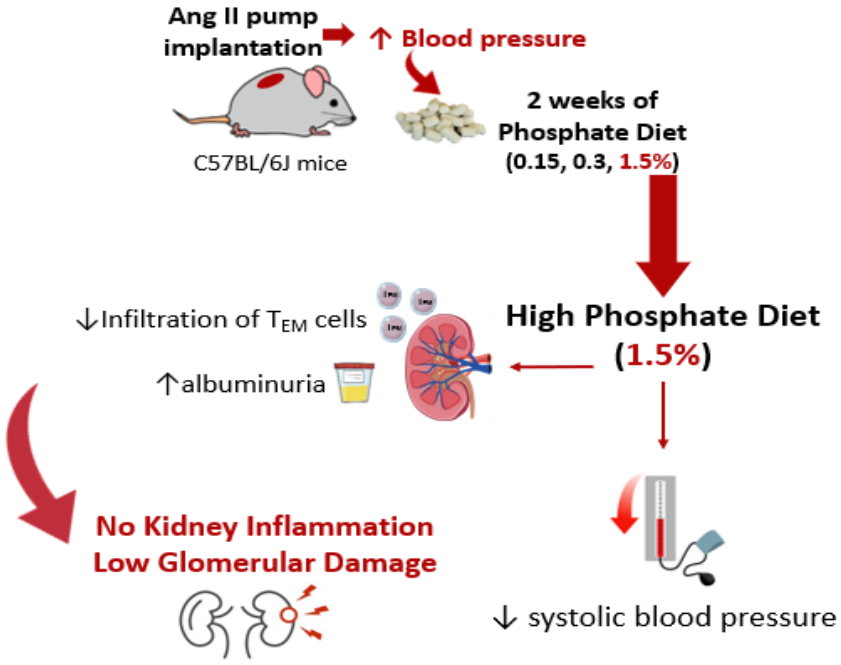

Figure 21. Working Model. Ang-II infused mice fed high dietary phosphate exhibit a decrease in blood pressure, reduced immune cell infiltration, and reduced glomerular damage.

CHAPTER 6

CONCLUSION

In conclusion, high dietary phosphate (1.5%P) administered after a hypertensive stimulus due to Ang II, decreases the SBP by 24.8 mmHg and the DBP by 19.32 mmHg. Hence, this decrease in BP prevented the infiltration of the immune cells in the kidneys, especially CD4+ and CD8+, therefore protecting it against inflammation that will be caused by hypertension. Therefore, extensive kidney damage in mice fed high phosphate was absent, suggesting that high dietary phosphate protects the kidneys from damage. The effect of the low dietary phosphate on blood pressure, inflammation, and kidney damage was not that significant compared to the high diet, thus not providing the protective desired outcome. Therefore, our hypothesis stating that dietary phosphate might reduce blood pressure, inflammation, and end-organ damage was truly verified for the high dietary phosphate concentration (1.5%P).

This project encompasses several limitations that need to be further studied to fully understand how and why high dietary phosphate decreases BP, inflammation, and damage. As we suggested, the decrease in BP is due to the coupled elimination of sodium and phosphate after reducing the expression of the sodium-dependent phosphate cotransport SLC34A1 and SLC34A3 in the kidneys; therefore, the expression of these channels should be confirmed as a mechanism. Moreover, the expression levels of the AT_1R and AT_2R should also be detected in order to check if the effects of Ang II are altered. In addition, detecting the levels of serum and urinary phosphate and sodium can also be tested to check the extent of their elimination. Finally, we should test the 24-h urine albumin: creatinine ratio at the beginning of the protocol, after 2 weeks of Ang II

infusion, and at the end of the protocol in order to compare and fully assert if the high ACR level is due to Ang II alone or the combination of Ang II and phosphate or due to partial phosphate elimination.

Whether high dietary phosphate is entirely beneficial and protective for all body organs and pathways requires more investigation and research to fully understand the whole picture of the 1.5% phosphate consequences.

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