EFFECTS OF POMEGRANATE EXTRACT SUPPLEMENTATION ON CARDIOVASCULAR DISEASE RISK AND PHYSICAL FUNCTION IN PATIENTS WITH CHRONIC RENAL FAILURE

BY

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DISSERTATION

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ABSTRACT

BACKGROUND: Patients with chronic kidney disease (CKD) undergoing maintenance hemodialysis therapy suffer from a variety of co-morbid conditions that greatly decrease physical function and increase cardiovascular disease (CVD) mortality. Oxidative stress has been implicated in increasing CVD risk and declining muscle function in hemodialysis patients, but little is known about the efficacy of antioxidant treatment in this population. A recent one year intervention in dialysis patients found that consumption of pomegranate juice, a rich source of polyphenol antioxidants, significantly lowered serum markers of inflammation and oxidative stress, reduced carotid atherosclerosis, and lowered the prevalence of hospitalizations due to infection. Despite these potential benefits, pomegranate juice is normally contraindicated in hemodialysis patients because its high potassium content may contribute to hyperkalemia-induced cardiac dysfunction. As a result, the efficacy of alternative antioxidant therapies needs to be investigated.

PURPOSE: The purpose of this study was to evaluate the effect of 6-month oral supplementation with a pomegranate extract containing a high concentration of antioxidant polyphenols, but low potassium content, on cardiovascular risk, physical function, oxidative stress and inflammation in hemodialysis patients.

METHODS: Thirty-three hemodialysis patients were recruited (20 men, 13 women; 54.3±2.1 years). Subjects were randomized to pomegranate (POM, n=16) or placebo (CON, n=17) group. At baseline and 6 months following the start of the intervention, cardiovascular risk was assessed by measuring arterial structure and function using a combination of vascular ultrasound and arterial tonometry, as well as circulating markers of oxidative stress and inflammation. In addition, a variety of tests were used to assess muscle strength and physical function.

RESULTS: Systolic and diastolic blood pressure (BP) were reduced by 24.2±13.7 mmHg and 10.9±5.3 mmHg, respectively, in POM ($p<0.05$), but did not change in CON. However, the BP differences in the POM group were no longer significant after controlling for baseline BP levels. Paraoxonase-1 activity, a measure of antioxidant capacity, increased by 26.6% ($p<0.05$) in POM, compared to no significant change in CON. However, pomegranate supplementation had no effect on other markers of CVD risk (e.g., β stiffness index, augmentation index, pulse wave velocity, or carotid intima-media thickness (CIMT)), serum markers of inflammation and oxidative stress, or measures of physical function and muscle strength.

CONCLUSION: This data suggests that while pomegranate extract supplementation may reduce blood pressure in hemodialysis patients, it does not improve other markers of cardiovascular risk, physical function or muscle strength.

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CHAPTER 1

INTRODUCTION

The prevalence of chronic kidney disease (CKD) in the United States has increased dramatically in the past decade. In particular, the prevalence of advanced kidney disease, defined as end-stage renal disease or kidney failure (CKD stage 5) treated with dialysis or transplantation, increased by nearly 20% in the US in the decade following 2000[1]. Compared to age- and gender-matched healthy controls , patients with end-stage renal disease treated with hemodialysis have a 10- to 30-fold increased risk of cardiovascular disease (CVD) mortality, which constitutes 58% of all-cause deaths in this population. Most patients with impaired renal function die of CVD rather than progress to renal failure, and the risk increases as renal function worsens[2-4]. Patients treated with hemodialysis also have greatly reduced levels of physical functioning. Muscle catabolism and wasting are especially common and lead to reduced muscle strength. Physical inactivity exacerbates these functional declines, and also promotes CVD. This cycle of disease and disability greatly reduces quality of life and increases mortality rates in CKD patients[5].

Excessive oxidative stress is believed to underlie many of the cardiovascular complications in this population, including increases in carotid intima-media thickness (CIMT), arterial stiffness, atherosclerosis, vascular calcification, and left ventricular hypertrophy (LVH)[6-8]. In addition, studies have shown that increased oxidative stress contributes to inflammation which promotes skeletal muscle protein catabolism and atrophy in patients with kidney failure[4, 9]. This suggests that antioxidant therapy could be efficacious in CKD patients, potentially to attenuate declines in both cardiovascular disease risk and muscle function.

In most populations, studies examining the effects of antioxidants (e.g., vitamin C, E, and β-carotene) on CVD risk have been disappointing[10]. In contrast, several studies in hemodialysis patients have shown that antioxidants may be effective in reducing CVD events[11, 12]. This indicates that antioxidant therapy may be more efficacious in hemodialysis patients than in other populations, possibly due to their excessive oxidative stress or other unique characteristics of the disease. However, few studies have examined this question.

Although the efficacy of antioxidant supplementation for primary or secondary prevention of muscle atrophy or dysfunction is limited, recent research indicates that novel antioxidants such as N-acetylcysteine[13], as well as isolated polyphenolic compounds[14, 15] which have higher antioxidant activity than standard supplements, can indeed improve muscle strength or reduce muscle catabolism. In particular, a recent study by Trombold et al.[14] found that 9 days of

pomegranate extract supplementation improved the recovery of isometric strength after eccentric exercise in healthy males. However, no studies to date have assessed the effects of antioxidant supplementation, or pomegranate specifically, on muscle strength or function in CKD patients.

Pomegranate fruit is enriched in polyphenols with high antioxidant capacities. Pomegranate juice was recently shown to improve CVD risk in patients with carotid artery stenosis by reducing CIMT in association with reductions in blood pressure and serum oxidative stress[16], and also has anti-inflammatory effects[17, 18]. In addition, a recent study in hemodialysis patients found that consumption of a very modest amount of pomegranate juice (~100ml/day) 3 days per week for one year significantly reduced markers of inflammation and oxidative stress, carotid atherosclerosis, and hospitalizations due to infection[19]. However, the patients in this study were required to consume the pomegranate juice immediately prior to their dialysis sessions so that the high potassium load from the juice could be removed in the dialysate. A potentially efficacious alternative strategy is to supplement patients with a pomegranate extract containing abundant antioxidant polyphenols, but only trace amounts of potassium.

The objective of this research was to determine if daily oral supplementation with a pomegranate extract can lower CVD risk and attenuate declines in physical function in hemodialysis patients. To examine this, we conducted a **double-blind, placebo controlled clinical trial** in which 33 patients receiving maintenance hemodialysis therapy were randomized

to the following groups for 6 months: 1) Control/placebo (CON; N=17), or 2) Daily supplementation with a 1,000mg capsule of a pomegranate extract (POM; N=16). We hypothesized that POM supplementation would: 1) reduce systemic markers of inflammation and oxidative stress; 2) reduce arterial stiffness and CIMT; and 3) attenuate declines in physical function and strength. Results from this study will help determine if pomegranate extract is an efficacious means of preventing the development and progression of CKD co-morbidities.

CHAPTER 2

LITERATURE REVIEW

2.1 Chronic Kidney Disease: Consequences of a Growing Epidemic

The prevalence of chronic kidney disease (CKD) in the United States has increased dramatically in the past decade. In particular, the incidence of advanced kidney disease, defined as kidney failure (glomerular filtration rate (GFR) < 15 ml/min/1.73 m^2 body-surface area) treated with dialysis or transplantation, increased in incidence by 43% in the United States in the decade following 1991[20]. Advanced CKD is associated with a variety of metabolic disturbances that increase morbidity and mortality. In addition, oxidative stress/antioxidant imbalance, muscle wasting, and cardiovascular complications are especially common, and these co-morbidities greatly reduce physical function and quality of life in dialysis patients. As a result, new strategies aimed at improving the health and quality of life of dialysis patients are needed.

2.2 Cardiovascular Disease – The Leading Cause of Death in CKD Patients

CVD is the leading cause of death in CKD patients, and the risk increases as the severity of the CKD increases. Greater than 58% of premature deaths in dialysis patients have been

attributable to CVD (reviewed in[21]). This increased CVD mortality is due to a variety of inter-related factors, including excessive vascular calcification (VC)[22-24]. VC is part of a remodeling of the vascular wall in CVD that promotes arterial and aortic stiffness[25], as well as other deleterious functional outcomes, including increases in arterial wall intima-media thickness, endothelial dysfunction, changes in atherosclerotic plaque stability, and a variety of clinical end points, such as LVH[25, 26].

Studies have demonstrated that arterial stiffness is associated with LVH and an independent predictor of all-cause and cardiovascular mortality in dialysis patients[27-30]. In patients with stage 5 CKD, with the progression of anemia, a decrease in the blood viscosity, and the creation of arteriovenous (AV) shunt, the peripheral resistances are most frequently normal or lower. Thus, the principal pressure factors opposing LV ejection are arterial stiffness and early return of wave reflections[27, 31] . In patients with CKD, arterial function alterations are associated with arterial remodeling including intima-media hypertrophy of arteries[26], characterized by stiffening of the arteries. Arterial stiffness is a major determinant of left ventricular pressure overload and of abnormal coronary perfusion in this population[32].

Traditional pharmacological therapies used to treat or prevent CVD in patients with normal renal function (e.g., lipid lowering medications) are generally less effective in CKD patients, possibly due to the excessive CVD risk observed in this population which is not fully accounted

for by traditional CVD risk factors[33]. A part of the extra risk is also attributable to renal-specific risk factors, related to uremia, including inflammation, malnutrition, and oxidative stress[34]. Elevated plasma concentration of pro-inflammatory cytokines and of the acute-phase reactant c-reactive protein (CRP) are among the strongest risk predictors of subsequent cardiovascular morbidity and mortality in uremic patients[35-37].

2.3 Muscle Catabolism in Dialysis Patients

CKD Patients treated with hemodialysis are characterized by a severely limited exercise capacity[38], reduced skeletal muscle strength and loss of lean muscle mass[39]. Evidence suggests that muscle atrophy in dialysis patients is the result of an enhanced muscle proteolysis[40], and it is associated with several metabolic abnormalities that stimulate protein degradation[41]. Metabolic acidosis, commonly occurring in dialysis patients, causes loss of muscle mass by activating irreversible oxidation of essential, branched-chain amino acids [42]. This suggests that reduction in muscle wasting might be achieved with correction of acidosis or the resulting oxidative stress in dialysis patients[43].

Insulin resistance, another common complication in dialysis patients, could also accelerate muscle atrophy by activating the ubiquitin-proteasome system in muscle [44]. The (ATP)-dependent ubiquitin-proteasome proteolytic pathway is considered to be the major

process to remove damaged proteins produced by genetic alterations or by oxidative stress. Evidence for this includes the presence of higher levels of the transcription of ubiquitin and subunits of the proteasome[44-46]. Two ubiquitin ligases, atrogin-1 (also known as MAFbx) and MuRF-1, are found specifically in muscle, and their expression increases dramatically in catabolic states, causing loss of muscle protein[47]. Additional evidence linking the ubiquitin-proteasome proteolytic pathway to protein degradation in catabolism is the finding that the increase in protein degradation in the muscle of rats with CKD can be blocked by inhibitors of the proteasome[45, 46].

A third mechanism causing loss of protein stores is that the dialysis procedure itself stimulates protein degradation in muscle [48]. The most convincing evidence that suggests increased protein breakdown induced by hemodialysis was provided by Gutierrez et al.[49]. These investigators showed increased amino acid release from the leg during sham hemodialysis in normal subjects using a bioincompatible dialyzer, but not with biocompatible membranes. Furthermore, evidence indicates that levels of circulating inflammatory cytokines are elevated during kidney disease progression, and these cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), have been suggested to be responsible for muscle catabolism[50, 51] via up-regulation of the ubiquitin-proteasome proteolytic pathway [52].

Finally, oxidative stress, an imbalance between free radical formation and antioxidant status, is the major contributor to increased levels of circulating inflammatory cytokines and associated with accelerated protein catabolism in dialysis patients by up-regulating elements of the ubiquitin conjugation pathway[53]. In summary, there are several oxidative balance abnormalities in skeletal muscle of CKD patients that can stimulate protein degradation and lead to loss of muscle mass.

2.4 Chronic Inflammation and Oxidative Stress in CKD

As increased oxidative stress and inflammation are both common features of CKD, it has been suggested that they may be mechanistically linked[54-56]. Excessive oxidative stress is believed to be involved in triggering the inflammatory process by elevating circulating levels of acute phase proteins such as CRP and pro-inflammatory cytokines (especially IL-6 and TNF-α)[7, 36, 57], and is also a significant contributor to the accelerated pathology associated with CKD[58-60].

Oxidative stress, resulting from the imbalance of reactive oxygen species (ROS) production and anti-oxidant defense mechanisms, is correlated with the level of renal dysfunction among patients with CKD[7, 61]. The increased oxidative stress in CKD patients is mainly attributed to the retention of oxidized solute due to the loss of filtration function of the kidney, and the increase in oxidative stress becomes more pronounced as renal function deteriorates[58, 62]. In addition,

blood-membrane interaction during hemodialysis triggers circulating neutrophils to produce high amounts of ROS, including superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous acid[63]. Therefore, hemodialysis treatment has been proposed to impose additional oxidative stress on patients with end-stage renal disease because of the imbalance between ROS production and antioxidant defense mechanisms.

ROS, which are generated both physiologically and pathologically, cause damage to cellular constituents, including membrane lipids, proteins, and DNA. In recent studies, the plasma concentration of oxidative stress markers such as malondialdehyde (MDA), a byproduct of the peroxidation of polyunsaturated fatty acids, and oxidized low-density lipoprotein (ox-LDL) were noted to be significantly increased in dialysis patients and have also been used to predict mortality in uremic patients[64-66]. Studies also demonstrate significantly elevated serum concentrations of CRP and F_2-isoprostane, a biomarker of lipid oxidation, in hemodialysis patients compared with patients with normal kidney function[67, 68].

Plasma levels of protein carbonyls and advanced oxidation products of proteins (AOPP), an index of oxidant-mediated protein damage, are significantly increased in uremic patients[69, 70]. In addition, AOPP levels were found closely related to advanced glycation end products (AGEs) and monocyte activation markers in dialysis patients[71]. Finally, AOPP was identified as a marker of oxidative stress and a potent trigger of the monocyte respiratory burst in this population.

In contrast to lipids and proteins, the reactions of DNA with various oxidants have not been well studied in hemodialysis patients. Phagocytes are activated after contact with bioincompatible dialyzer membranes, so, leukocytes of patients undergoing chronic hemodialysis may be useful for monitoring changes in the level of cellular DNA oxidation as they are not only the source, but also the target of endogenously produced ROS and free radicals. Among the many types of modifications induced by ROS, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most abundant oxidative products of DNA[72, 73], and is capable of reflecting extremely low levels of oxidative DNA damage and is therefore useful as a surrogate marker of oxidative stress in hemodialysis patients[74]. Hemodialysis patients exhibit increased oxidative DNA damage, compared to age- and sex-matched healthy individuals. Furthermore, endothelial function has been shown to be negatively correlated with 8-OHdG in hemodialysis patients[75].

2.5 Impairment in Antioxidant Defense System

Several intracellular and extracellular antioxidant systems have evolved to detoxify specific free radicals and other oxidants. However, antioxidant mechanisms that serve as a safeguard against ROS seem to be impaired in hemodialysis patients, and deficiencies in different components, including reduced levels of enzymatic antioxidants (e.g., superoxide dismutase

(SOD), glutathione peroxidase (GSH-Px), and catalase (CAT))[76, 77] and extracellular antioxidant defense elements (such as reduced glutathione (GSH))[78, 79]. Furthermore, decreased levels of hydrophilic and lipophilic antioxidant vitamins in uremia have also been observed[34, 80, 81]; if antioxidant defense is compromised, elevated ROS formation in the body will lead to increased damage.

2.6 Effects of Single Hemodialysis Sessions

Hemodialysis is a non-selective process clearing solute solely based on molecular weight, a sieving property of the membrane and protein bound capacity. Consequently, hemodialysis induces solute losses, including antioxidants. In addition, hemodialysis per se has been suggested to induce oxidative stress, with ROS being generated on the surface of dialysis membranes[82-84]. Indeed, it has been well documented that even a single session of hemodialysis significantly increases lipid peroxides and decreases antioxidants[85-88].

Contribution of the dialyzer membrane in the production of inflammation and ROS has been studied in acute hemodialysis conditions by comparing cellulosic (cuprophane) and synthetic (polysulfone) dialyzer membranes[63, 89]. Plasma levels of inflammatory cytokines (such as IL-6 and TNF-α) are elevated in dialysis patients, and their expression was exacerbated further even after a single dialysis treatment[90, 91]. Although there are controversial results in the literature

indicating that hemodialysis could improve lipid profiles in hemodialysis patients[92], others have indicated an increase in lipid peroxidation during hemodialysis[89, 93]. The majority of ROS generated during a single dialysis session is hydrogen peroxide[91] which causes subsequently lipid peroxidation, which enhances the susceptibility of LDL oxidation and is recognized as the major initiating event in the genesis of atherosclerosis. Besides the alterations in lipid levels, hemodialysis treatment also contributes to increased oxidative stress at the protein level[89, 94].

ROS can not only inactivate mitochondrial enzymes or damage lipid, protein and DNA, but also actively react with nitric oxide (NO), a principal endothelial-derived relaxing factor, to yield the powerful oxidant, peroxynitrite ($ONOO^-$). The inactivation of NO by ROS creates NO deficiency and could play a role in the arterial stiffness.

Interestingly, NO production during hemodialysis is increased rather than decreased[95-98]. Most studies indicate that NO increases early during dialysis, possibly due to shear stress or leukocyte activation by the dialysis membrane[82, 83, 95, 96, 99]; however, a significant drop in NO levels usually occurs at the end of dialysis[98, 100], probably due to its elimination during the dialysis session, and returned to high levels between the sessions. The increased NO production is, however, attacked by superoxide and other ROS released as a result of bioincompatibility of the dialyzer membrane. This results in formation of $ONOO^-$ which release hydroxyl radicals. The hydroxyl radicals in the presence of fatty acids released by the lipolytic effect of heparin

perpetuate the oxidative stress and cause the $ONOO^-$ diverted toward lipid peroxidation. As a result, hemodialysis can be seen as a situation of continuous production of NO, with simultaneous inactivation by ROS, leading to endothelial dysfunction[101].

Given the intimate causal interaction between oxidative stress and inflammation, interventions that aim to alleviate oxidative stress can potentially ameliorate inflammation. Several clinical studies have attempted to treat oxidative stress and inflammation in this population by a variety of antioxidant regimens. Many of these studies have employed vitamin E or C given either orally or parenterally[10, 79, 102]; however, these studies have produced conflicting results and lack of acute responses to single antioxidant intervention. These data may be partially explained by a vitamin C-related increase in catalytic iron and oxalate that favors prooxidant activity; different doses and route of antioxidant administration and different markers of oxidative stress may also help explain the results.

2.7 Oxidative Stress, Antioxidants and CVD in CKD

Elevated oxidative stress is a common feature in CKD patients that likely contributes to their excessive CVD risk[34, 103-105]. Increased oxidative stress results in vascular injury and a variety of functional CVD outcomes, including increases in arterial stiffness[106], vascular dysfunction[107], CIMT[108], and vascular calcification[109](Figure 1).

Superoxide anion, a primary ROS which is generated from a one-electron reduction of molecular oxygen, may inactivate nitric oxide (NO) and diminish its bioavailability, thus ultimately lead to endothelial dysfunction[110, 111]. Endothelial dysfunction is especially important in renal disease, given the nature of accelerated risk of CVD in this population.

Because inflammation, oxidative stress and the pathogenesis of CVD are closely intertwined, and oxidation products may mediate inflammation in CKD patients, antioxidant supplementation is of particular interest[12, 81]. Due to the excessive oxidative stress in patients with advanced CKD, antioxidants may be particularly effective in this population.

Despite a demonstrated lack of efficacy of vitamin E in most clinical trials, studies recently showed that oral vitamin E supplementation reduced the incidence of myocardial infarction[11] and improved oxidative stress[79] in CKD patients. Similarly, Tepel et al showed that twice daily supplementation with 600mg of the antioxidant N-Acetylcysteine (NAC) reduced cardiovascular endpoints by 40% in CKD patients[12]. Yet, few studies have linked the reduced oxidative stress to the functional cardiovascular outcomes in CKD patients.

Most previous trials assessing the efficacy of antioxidants have used single compounds such as β-carotene, vitamin A, vitamin C, vitamin E, and selenium, each of which has rather limited substrate specificity. Also, antioxidants vary significantly in their capacity to scavenge free radicals, chelate transition metal ions, and/or inhibit oxygenase/oxidase activity. A more

efficacious approach may be to use combinations of antioxidants with diverse substrate specificities.

2.8 Effects of Antioxidants on Muscular Performance

Several lines of evidence link ROS to muscle atrophy via reductive-oxidative (redox) control of proteolysis. Importantly, a growing number of studies suggest that antioxidants can serve as therapeutic agents in delaying the rate of muscle atrophy.

Abundant research demonstrated that oxidative stress contributes to muscle atrophy, and this phenomenon could be delayed by exogenous antioxidants.

A recent cross-sectional study supports the hypothesis that oxidative stress is associated with the loss of muscle mass in older adults[112]. Findings from this study showed that higher plasma concentrations of antioxidants were associated with reduced risk of low grip, hip, and knee strength. Similarly, reduced muscle damage in claudicants was found with daily administration of vitamin E (200mg) and vitamin C (500 mg) for 4 weeks[113]. These findings suggest that antioxidants may play a role in preventing oxidative protein modification in muscle and preserving muscle function. The work of Appell et al. revealed that there was less oxidative stress in the vitamin E supplemented muscles than the control group in rats, and eight days of immobilization lead to a 35% atrophy, while with vitamin E the muscles atrophied only by

12%[114]. This difference can be attributed to the action of vitamin E as a scavenger for free radicals and, on the other hand, to an atrophy promoting effect of oxidative stress.

Another study also showed that Trolox attenuates the mechanical ventilation-induced diaphragmatic contractility deficit. Mechanical ventilation is associated with a rapid onset of protein oxidation in diaphragm fibers, and has been directly linked to activation of the ubiquitin–proteasome system of proteolysis. However, diaphragmatic proteolysis did not differ between controls and mechanical ventilation-Trolox animals. Moreover, proteasome activity in the diaphragm was elevated in the mechanical ventilation animals, while Trolox treatment attenuated this mechanical ventilation-induced rise in protease activity[115].

Hauer et al. [13] performed a randomized, placebo-controlled trial in the elderly with 13 weeks of 200 mg N-Acetylcysteine (NAC) supplementation. NAC is a precursor for glutathione, an important antioxidant that protects cells against oxidative stress. NAC administration resulted in improvement in muscle strength and decrease in TNF-α level, and suggesting a potential anti-inflammatory role. In addition, a recent study showed that supplementation with ellagitannins from pomegranate extract significantly improved isometric strength 2-3 days following damaging eccentric elbow flexion exercise in recreationally active males[14, 15]. The recovery of strength during the 24- to 48-h period was more rapid, and strength was significantly higher in the subjects who consumed 500mL of pomegranate extract beverage, compared to the

placebo group. Together, these findings suggest that antioxidant supplementation, including pomegranate extract, may improve muscular performance. Furthermore, the effect of antioxidant supplementation on the muscular performance might be mediated through changes in the levels of inflammation and oxidative stress.

2.9 Pomegranate – A Rich Source of Potent Antioxidants

A specific source of antioxidants with therapeutic potential in dialysis patients is pomegranate fruit (*Punica granatum L.*), which contains a variety of polyphenols and other antioxidants, including punicalagin, anthocyanins, ellagic acid and gallic acid, that have particularly potent and diverse antioxidant capacities[116, 117]. The antioxidant capacity of pomegranate juice was shown to be three times higher than that of red wine and green tea, based on the evaluation of the free-radical scavenging and iron reducing capacity of the juices[116].

Pomegranate also has been reported to inhibit lipid peroxidation[118], and pomegranate showed better protection than vitamin E and NAC against oxidative stress[119]. In addition, pomegranate may inhibit inflammation-stimulated c-Jun N-terminal kinases (JNK)- and extracellular signal-regulated kinase (ERK)- mitogen-activated protein kinase (MAPK) activation through the suppression of nuclear factor kappa-light-chain-enhancer of activated B cells (NK-kB) activity[17], increasing endothelial NO synthase expression[120, 121], and reducing

atherosclerotic lesions[122]. But unlike most studies using single antioxidants, several small clinical trials indicate that pomegranate juice or extracts also help reduce CVD risk in humans.

Recently, Aviram et al. showed that 1 year of pomegranate juice consumption by patients with severe internal carotid artery stenosis reduced CIMT by 30%, increased antioxidant capacity by 130%, and substantially inhibited LDL-lipid peroxidation[16]. In a follow-up study with subjects at moderate risk for CHD, 1 year of pomegranate juice consumption attenuated anterior wall CIMT progression, which was thicker at baseline than posterior wall CIMT, while pomegranate juice showed little or no effect on progression rates in the posterior wall CIMT[123]. Furthermore, in the subgroup of patients with the greatest risk of CVD, those in the pomegranate juice group had less CIMT progression versus control subjects. This finding suggests that pomegranate may be most efficacious in subjects with the greater oxidative stress and/or CVD risk. In addition, pomegranate juice and extract consumption in diabetics resulted in a significant improvement in markers of oxidative stress and atherogenesis without worsening their diabetic parameters[124, 125]. Another potential benefit of pomegranate is that it has been shown to increase endogenous antioxidant activity[16], suggesting another potential mechanism for its cardioprotective effects.

Evidence for the clinical benefits of pomegranate juice in the patients undergoing hemodialysis was recently reported by Shema-Didi et al.[19] Hemodialysis patients who consumed

100 mL of pomegranate juice 3 times a week during dialysis sessions for 12 months had significant reductions in neutrophil, IL-6 and oxidized fibrinogen, while the placebo group had no change in levels of any of the markers. In addition, hospitalizations for cardiovascular causes were reduced by 40% in patients randomized to pomegranate juice, but did not change in the placebo group. However, pomegranate juice contains high concentrations of potassium, creating a potential for potassium overload in dialysis patients. No studies to date have examined the efficacy of pomegranate extract, which contains much lower concentrations of potassium than juice, on CVD risk or physical function in this population.

Taken together, these data indicate that antioxidant intervention may be more efficacious in patients with excessive oxidative stress, such as patients undergoing hemodialysis treatment. In addition, pomegranate extracts may be more effective than single antioxidants in reducing CVD risk and attenuating muscle wasting in clinical populations.

2.10 Safety and Drug Interactions

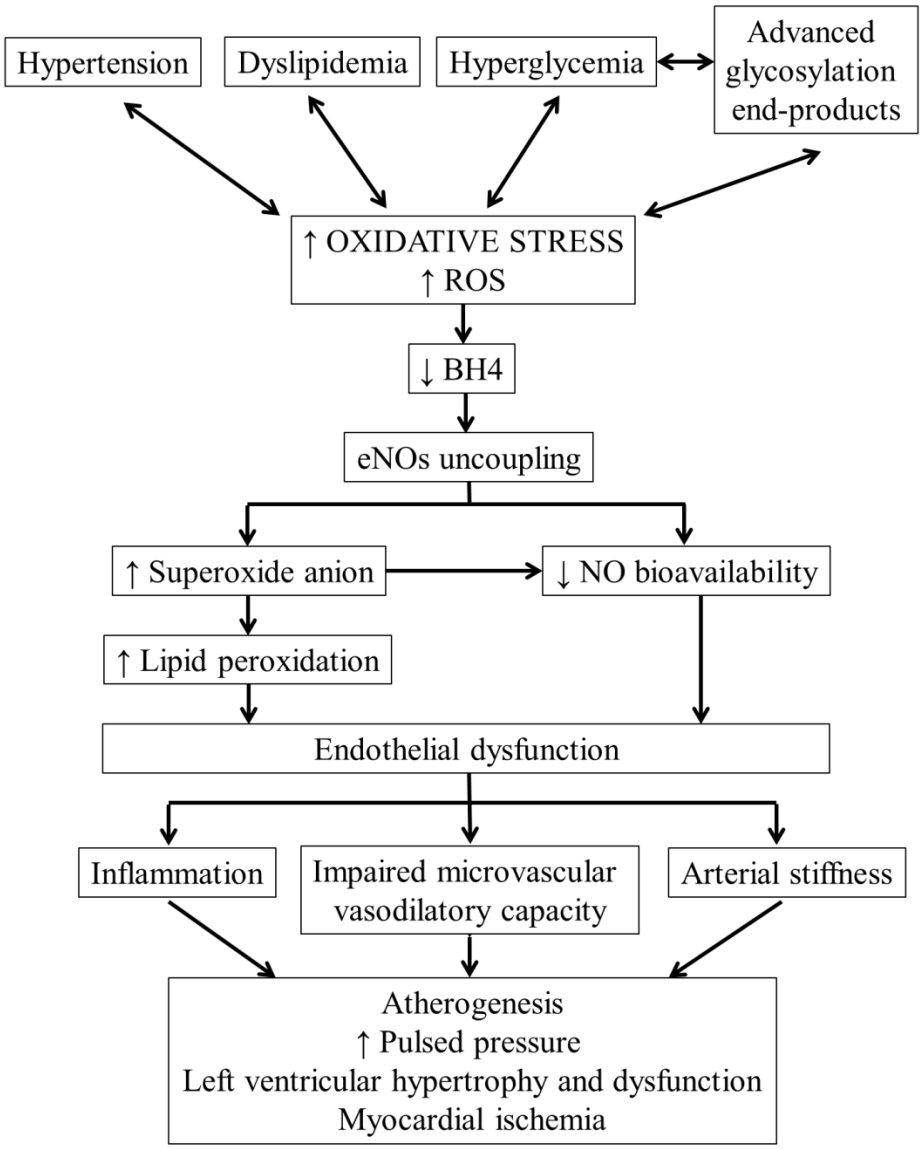
Drug-dietary supplement interactions have been a concern in clinical research, and conflicting data regarding pomegranate's impact on hepatic P450 2C9 (CYP2C9) enzymes have been published. Case reports of patients experiencing potential drug toxicities while drinking pomegranate juice have been reported. One patient on warfarin had an increase prothrombin time

after introducing pomegranate juice into the diet[126]. Another subject experienced rhabdomyolysis after drinking pomegranate while on rosuvastatin and zetia combination therapy[127]. Both case reports, however, were confounded by preexisting conditions that may have explained observed results. A recent study investigated the effect of pomegranate juice and pomegranate extracts on inhibiting human CYP2C9 activity by using flurbiprofen as a probe compound to profile CYP2C9[128]. This study showed that pomegranate juice and pomegranate extracts inhibit CYP2C9 activity *in vitro*, but not *in vivo*. This finding indicates that subjects on CYP2C9-related medications can consume pomegranate juice or extract with little risk of drug-supplements interaction.

Figure Legends

Figure 1 Mechanisms responsible for oxidative stress-induced increases in cardiovascular disease risk (modified from[105, 129]). Cardiovascular disease is one of the major complications of CKD patients undergoing dialysis. Other common co-morbidities in this population, such as hypertension, dyslipidemia, hyperglycemia, are centrally linked to increased oxidative stress and reactive oxygen species (ROS). Elevated oxidative stress and ROS can cause oxidation of tetrahydrobiopterin (BH4), a cofactor for the production of nitric oxide (NO) by endothelial nitric oxide synthases (eNOs), and consequently lead to uncoupling of eNOS. This reaction generates superoxide anion and causes decreased NO production and bioavailablilty. Superoxide anion can cause lipid peroxidation, accumulation of which contributes to endothelial dysfunction. Nitric oxide (NO) has been shown to be a potent vasodilator. Oxidative stress-induced reductions in NO production and bioavailability also promote endothelial dysfunction. This can impair microvascular vasodilatory capacity, elevate inflammatory levels, and increase arterial stiffness. Furthermore, these responses result in the development of cardiovascular complications, including atherogenesis, left ventricular hypertrophy and dysfunction, and myocardial ischemia.

Figure 1 Mechanisms responsible for oxidative stress-induced increases in cardiovascular disease risk (modified from[105, 129]).



ROS, reactive oxygen species; BH4, tetrahydrobiopterin; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

CHAPTER 3

RESEARCH DESIGN AND METHODS

3.1 Study Overview

Patients with renal failure receiving maintenance hemodialysis therapy (CKD stage 5) at local dialysis clinics were recruited and randomly assigned to one of two groups for 6 months: 1) Usual care/control (CON; N=14); or 2) Daily oral supplementation with a purified pomegranate extract (POM; N=13). Patients in the POM group ingested a 1,000mg capsule of a pomegranate extract every day for 6 months. Patients in the CON group ingested a placebo capsule using the same protocol. At baseline and 6 months following the start of the supplementation period, all patients had blood collected to measure markers related to inflammation, oxidative stress, and antioxidant capacity. The subjects also came in for clinical testing at each time point to measure factors related to cardiovascular disease (CVD) risk and physical function. A study timeline is shown in Figure 2.

3.2 Subjects, Recruitment, Screening, and Selection

Thirty-three patients with CKD stage 5 (GFR < 15 ml/minute/1.73 m^2) receiving hemodialysis therapy for more than 3 months were recruited on a rolling basis from the hemodialysis clinics in Champaign and Chicago, IL. Patients were recruited through advertisements that were placed in the clinics, as well as through brochures provided to subjects by the medical staff and research members. Those expressing an interest in the study signed a form indicating they would like to be contacted by a member of the research staff to acquire more information. During the screening, subjects were informed of the study purpose, the risks and benefits pertaining to their participation, the randomization process, the proposed testing, and the necessary time commitment. Patients were screened for eligibility by administering a medical history questionnaire (see exclusion criteria below), and an informed consent document was provided for each individual to sign upon their agreement to participate. A physician clearance for each subject was signed by their nephrologists. All protocols were approved by the Institutional Review Board of the University of Illinois.

3.3 Inclusion/Exclusion Criteria

Inclusion/exclusion criteria for volunteers include the following: 1) Subjects must be diagnosed with CKD stage 5, and had been receiving hemodialysis treatment 3 times per week for ≥ 3 months. This criteria was chosen based on research indicating oxidative stress increases

as renal function deteriorates[130], and our working hypothesis is that pomegranate supplementation would be most efficacious in individuals with excessive oxidative stress. 2) Subjects must be ≥ 30 years of age. As previous studies have shown arterial stiffness increases significantly after 30 years of age in CKD patients (unpublished observations from our laboratory), and limiting the recruiting pool to those ≥ 30 years of age will reduce the number of recruited subjects with little or undetectable arterial stiffness. 3) Subjects must be willing to be randomized to the control or intervention groups. 4) Subjects must receive medical clearance from their primary care physician to participate. 5) Subjects must NOT be consuming any form of antioxidant supplementation.

3.4 Group Assignment

Following recruitment, screening, and baseline testing (see below), 33 eligible subjects were randomized to the CON or POM groups as described below. Both the subjects and study personnel involved in data collection were blinded to the group assignment. We used a simple randomization procedure as follows: the eligible subjects were randomly sorted into pairs. In each pair, a random permutation of the subjects is used to assign them to the two groups (CON or POM). Under this randomization procedure, each subject has equal probability of being assigned to each group.

3.5 Intervention Protocol

Patients in the POM group ingested a 1,000mg capsule of a purified pomegranate polyphenol extract (POMx™, POM Wonderful, Inc. Los Angeles, CA; http://www.pompills.com/pills/product_pills.aspx) 7 days a week for 6 months. POMx pills are a concentrated blend of polyphenols extracted from whole pomegranates that have been safety tested by the Food and Drug Administration. The no-observed-adverse-effect level (NOAEL) of POMx was considered to be 1500 mg/kg of body weight (unpublished data, POM Wonderful LLC., 2006), which is approximately 100~125-fold more than what was being provided in this study. Research also showed that 1,000mg (610~755mg of gallic acid equivalents) of pomegranate extract is sufficient to provide antioxidant effects through a reduction in thiobarbituric acid reactive substances (TBARS), a byproduct of lipid peroxidation, in overweight subjects[131].

POMx is derived from pomegranates grown in California (Paramount Farms). POMx is prepared from inner and outer pomegranate peels, seeds and juice, pressed to produce a powder with a high concentration of polyphenols. The pomegranate extract is 98% (980 mg) of the pill's active ingredient. The extract is composed of 5.7% ellagic acid, 25.6% punicalin/punicalagin, and 68.4% other polyphenols. The remaining 2% of the drug is magnesium stearate. The

matching placebo pills contain 0mg of gallic acid equivalents, and the ingredients include cellulose, caramel, beet root, magnesium stearate, and silicon (source: PomWonderful, unpublished data). The study drugs were stored at the Agricultural Engineering Sciences Building. Dry storage is required and shelf life is 18 months at or below 25˚C.

We chose to use the POMx pills instead of pomegranate juice because the juice contains significant amounts of potassium (430mg per 8 ounces or 12% of the recommended Daily Value for healthy adults), which is restricted in CKD patients, while the POMx pills have marginal levels (<0.998 mg per 1,000mg pill) of this mineral. Hyperkalemia, the condition of overabundance of potassium in the extracellular compartment, is common in patients with end-stage renal disease. Excess intake of potassium through dietary indiscretion and oral supplementation are the most common causes of hyperkalemia in dialysis patients. The effect of hyperkalemia on cardiac conductivity is its most feared clinical consequence, symptoms including slow pulse, irregular heartbeat, heart failure, and even sudden death. Severe hyperkalemia may also profoundly affect skeletal muscle, manifesting as motor weakness and parasthesia.

Individuals in the CON group ingested a non-caloric placebo capsule using the same protocol. Patients in both groups received a pill bottle containing a month's supply of capsules (POM or placebo) prior to the beginning of each month. They were asked to bring their pill

bottles to the dialysis clinics on a monthly basis so the number of remaining pills can be counted for compliance purposes. If a participant was non-compliant, defined as completing less than 75% of the available supplements (and does not respond to repeated attempts to increase consumption), their data was not included in the analysis.

3.6 Baseline Testing and Measurements

3.6.1 Anthropometric Measures

Barefoot standing height was measured to the nearest 0.1 cm with a stadiometer and body weight was measured on a balance scale with shoes and superfluous outer garments (e.g., jackets) removed. Waist circumference was measured as the minimum circumference between the top of the iliac crest and the distal end of the rib cage along the mid-axillary line. An additional measure of waist circumference was also taken at the umbilicus, and the average of the 2 waist circumference measurements was used in all analysis. Hip circumference was measured at the maximal hip circumference. All measurements for a given participant were taken in triplicate and averaged.

3.6.2 Blood Chemistry

A small amount (20 – 30 ml) of blood was collected from each subject at baseline and 6 months for analyses described below. Plasma and serum from these extra samples was collected by centrifugation, divided into 350ul aliquots, and stored at -80°C until analyzed.

3.6.2.1 Oxidative Stress Biomarkers

Advanced oxidation protein products (AOPP) are uremic toxins that are a marker of protein oxidative stress created through the reaction of plasma proteins with chlorinated oxidants, such as chloramines or hypochlorous acid. Concentration of AOPP in the plasma was determined using the semi-automated method as previously described[69]. Briefly, AOPP was measured by spectrophotometry on a microplate reader (GENios Pro, Tecan, Männedorf, Switzerland) and calibrated with chloramine-T (Sigma, St. Louis, MO) solutions that in the presence of potassium iodide absorb at 340 nm. In test wells, 200 µl of plasma diluted 1/5 in PBS was placed on a 96-well microtiter plate, and 20 µl of acetic acid was added. In standard wells, 10 µl of 1.16 M potassium iodide (Sigma, St. Louis, MO) was added to 200 µl of chloramine-T solution (0–400 µmol/liter) followed by 20 µl of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm on the microplate reader against a blank containing 200 µl of PBS, 10 µl of potassium iodide, and 20 µl of acetic acid. AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents.

Oxidized low-density lipoprotein (ox-LDL), a marker of lipoprotein-associated oxidative stress[132], was measured in triplicate using commercially available ELISA kits (Mercodia, Uppsala, Sweden).

8-hydroxy-2'-deoxyguanosine (8-OHdG) is produced by oxidative damage of DNA by reactive oxygen and nitrogen species. It serves as a biomarker of DNA oxidation, and was measured in triplicate using commercially available ELISA kits (Trevigen, Gaithersburg, MD).

3.6.2.2 Antioxidant Capacity

The Oxygen Radical Absorbance Capacity (ORAC) assay was performed according to published methods by Prior et al.[133] Fluorescein reacts with free radicals generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) yielding a non-fluorescent product. Loss of fluorescence was measured over time in fluorescent plate reader, FLx800tbi (Bio-Tek, Winooski, VT), at 37 °C and sensitivity 60. Readings were made with excitation 485 nm and emission 528 nm. The area under the curve (AUC) was calculated using the following equation:

$$AUC = 0.5 + f_1 / f_0 + f_i / f_0 + \ldots + 0.5\,(f_n / f_0)$$

$$\text{Net AUC} = AUC_{sample} - AUC_{blank}$$

Where AUC is area under the curve, f_1 is fluorescence of first reading (2 min); f_0 is fluorescence of reading time zero and f_n are n fluorescence readings. Areas under the curve were compared to a standard antioxidant, Trolox (vitamin E analog). Results were expressed as μM Trolox equivalents.

High-density lipoprotein (HDL) associated paraoxonase-1 (PON-1) is an antioxidant enzyme that protects lipoproteins from oxidation, and is considered a primary anti-atherosclerotic component of HDL[119, 134, 135]. Previous research has shown that pomegranate supplementation increases PON-1 activity in diabetic and healthy subjects[124, 136]. PON-1 activity, including arylesterase (monoesterase), paraoxonase (triesterase), and lactonase, was measured to assess serum antioxidant capacity as described[16, 137-139].

Lactonase, paraoxonase, and arylesterase activity was measured kinetically at 37°C using dihydrocoumarin, paraoxon, and phenyl acetate as a substrate, respectively. PON-1 activity was measured using a microplate reader (GENios Pro, Tecan, Männedorf, Switzerland), and the activity of lactonase, paraoxonase, and arylesterase was reported as units per milliliter, where 1 U is defined as 1 mmol of dihydrocoumarin, paraoxon, and phenyl acetate hydrolyzed per minute, respectively.

3.6.2.3 Markers of Inflammation

Serum CRP and IL-6 were measured in triplicate using commercially available ELISA kits (CRP:Alpco, Salem, NH ; IL-6: R&D Systems, Minneapolis, MN). To control for variation between runs, baseline and 6-month samples from each subject were analyzed simultaneously. Published intra-assay coefficients of variation were less than 5% for each assay.

3.6.3 Cardiovascular Disease Risk Measures

3.6.3.1 Blood Pressure

Brachial systolic (SBP) and diastolic (DBP) blood pressure was obtained using standard methods following a 10-minute period of quiet supine rest in a dimly lit room. Brachial blood pressure was measured using an automatic digital blood pressure monitor (Omron IntelliSense HEM-907XL, IL). All the BP measurements were repeated and the average of the two values, 1-minute apart, was recorded and used for analysis. If the values differed by ≥ 5mmHg, a third measurement was obtained and the two closest values were averaged. Mean arterial pressure was calculated by DBP + 1/3 (SBP-DBP).

3.6.3.2 Carotid Artery Stiffness

A combination of ultrasound imaging of the common carotid artery with simultaneous arterial tonometry of the carotid artery (for estimation of carotid blood pressure) allows for a

non-invasive determination of carotid arterial compliance. Carotid ultrasound images were obtained from the common carotid artery, 1-2 cm proximal to the carotid bifurcation using a 7-13 MHz linear array transducer with a sampling rate of 1,000 Hz. B-Mode and M-mode images were obtained and displayed simultaneously and automated wall tracking software was used to detect changes in lumen size as described above. Electronic calipers were applied to the arterial wall using the B-mode image and wall tracking is conducted using the M-mode image in real time. Changes in lumen size between systole and diastole is recorded for 12 seconds and an ensemble average beat is constructed from which measurements of arterial compliance and stiffness was conducted. β-stiffness index (β) was calculated with lumen size in systole and diastole. The calculation for β is shown below:

$$\beta = \text{In} (P_s - P_d) / (D_s - D_d)$$

P_s=systolic pressure, P_d=diastolic pressure, D_s = maximum vessel diameter, and D_d = minimum vessel diameter.

Carotid blood pressure was estimated using simultaneous arterial tonometry on the contralateral carotid artery. A high fidelity pencil probe strain gauge transducer (Millar Instruments, Huston, Texas) was interfaced with acquisition software (SphygmoCor, AtCor

Medical, Sydney, Australia). The carotid waveform was calibrated against brachial artery pressure to derive carotid artery pressure using standard transfer functions (SphygmoCor, AtCor Medical, Sydney, Australia).

3.6.3.3 Carotid Intima-Media Thickness (CIMT)

CIMT were measured using a high-resolution ultrasound system (Aloka Alpha 7, Japan) with a 7-13 MHz linear array transducer. The common carotid artery was imaged at the proximal 1-2 cm straight portion. The carotid bifurcation or bulb was used as a reference point to standardize the position of the CIMT measurements between baseline and final testing when possible. All images were analyzed in a 10mm window which is 10mm distal to the bulb using Carotid Analyzer (Medical Imaging Applications, LLC, Coralville, IA) software. The intima-media thickness was defined as the distance between the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface of the far wall of the carotid artery. All measurements and analysis were made by the same trained investigator blinded to group assignment.

3.6.3.4 Wave Reflection

Applanation tonometry was performed using a high-fidelity strain-gauge transducer (SphygmoCor; AtCor Medical, Sydney, Australia) on the radial artery to obtain pressure waveforms. Using a generalized validated transfer function[140], a central aortic pressure waveform was reconstructed from the radial artery pressure waveform. Augmentation index (AIx) was calculated as the ratio of amplitude of the pressure wave above its systolic shoulder (i.e., the difference between the early and late systolic peaks of the arterial waveform) to the total pulse pressure. The result was expressed as a percentage and was used as an index of aortic pressure wave–reflection intensity. Because AIx is influenced by HR[141], AIx values were also normalized to a HR of 75 bpm.

3.6.3.5 Aortic Pulse Wave Velocity (PWV)

PWV was measured following current guidelines[142]. Using the same high-fidelity strain-gauge transducer (Sphygmocor, AtCor Medical, Australia) as in the wave reflection measurements, pressure waveforms were taken first at the right common carotid artery and then at the right femoral artery. Consecutive waveforms were captured for a 10-s epoch. Simultaneous ECG gating, as a timing marker, was assessed via a 3-lead CM5 configuration and further used to obtain HR[143]. The foot of the pressure wave was identified automatically, removing potential observer bias, using an algorithm that detects the initial upstroke via a line tangent to the initial

systolic upstroke point of the pressure tracing and an intersecting horizontal line through the minimum point[144].

Distances from the suprasternal notch to the femoral artery and from the carotid artery to the suprasternal notch were measured as straight lines with a tape measure and recorded to the nearest mm. The distance from the carotid artery to the suprasternal notch was then subtracted from the distance between the suprasternal notch and femoral artery to account for differences in the direction of pulse wave propagation.

Aortic PWV was calculated from the distances between measurement points and the measured time delay between 10 proximal and distal waveforms. The peak of the R wave recorded from the ECG was used as a timing marker. Values obtained from the carotid to the femoral artery (PWV) were taken as an index of "central" arterial stiffness. Integral software assessed pulse wave quality (strength of pulse wave signal, pulse height variation, pulse length variation, and base line variation) and standard deviation of mean time differences (SphygmoCor, AtCor Medical, Sydney, Australia).

3.6.3.6 Echocardiography

Echocardiography was performed using a multifrequency (1.5-4.25 MHz) transthoracic transducer to assess parameters related to cardiac function and systemic vascular resistance. Left

ventricular stroke volume was assessed with the Simpson‘s biplane technique. Cardiac output was calculated by multiplying the stroke volume by the heart rate. Systemic vascular resistance was calculated by dividing the mean arterial pressure by the cardiac output. To minimize the effect of variations in fluid volume in hemodialysis patients, studies were performed 18-24 hours after a hemodialysis session at all testing timepoints.

3.6.4 Physical Performance Measures

3.6.4.1 Functional Fitness Testing

Subjects underwent a battery of tests to assess functional fitness, including: 1) *Chair Stand Test* where subjects were asked to stand up from a seated position as many times as possible within 30 seconds; 2) *Arm Curl Test* in which subjects were asked to complete as many arm curls as possible during 30 seconds using either a 5-pound dumbbell for females, an 8-pound dumbbell for males; 3) *Chair Sit and Reach Test* that asked subjects to reach forward with both arms to try and touch their extended leg to assess flexibility; 4) *Back Scratch Test* where subjects tried to touch their middle fingers behind their back to assess upper body flexibility; and 5) 8 *Foot Up-and-Go Test* in which subjects were asked to walk around a cone placed 8 feet away from their chair and back again during a timed trial.

3.6.4.2 Shuttle Walk Test

Each subject underwent a shuttle walk test to assess physical performance. This is a progressive test in which patients walk back and forth continuously on a 10-meter course. The walking is paced by beeps which are programmed - the subject should be at the end of the course by each beep. They maintain each speed for one minute and then the pace is increased. The speeds increase so that in each successive minute the speeds are as follows: 1.12, 1.54, 1.88, 2.26, 2.64, 3.02, 3.4, 3.78, 4.16, 4.54, 4.92, and 5.3 miles per hour. The patient continues until they do not reach the end of the 10-meter course by the beep and the total distance covered is calculated. Measures of physical performance such as this shuttle walk test are frequently used to assess function in older and diseased people instead of more objective measures of aerobic capacity such as VO_{2max} testing. This is due to functional limitations like muscle weakness and shortness of breath that prevent these individuals from achieving standard criteria used in assessment of these more objective tests[145]. This shuttle walk test is well established as a part of the guidelines for assessment of fitness in patients with chronic pulmonary disease[146], and is often preferred to the six-minute walk test because it is paced, and therefore more objective.

3.6.4.3 Muscle Strength Testing

A Biodex System 3 Pro dynamometer (Biodex Corp., Shirley, NY) was used to assess hamstring and quadriceps strength. The reliability and reproducibility of this device is well established.[147] The subject sat upright on the Biodex chair with the axis of the dynamometer corresponding to the knee joint axis. Once the patient was positioned, the shoulder, waist, thigh, and lower leg proximal to the ankle were secured with straps. To avoid substitution and compensation of other muscle groups, hip belts and a chest restraint were used to prevent hip flexion and extension. Isolated isokinetic muscle torque of the knee joint in flexion (hamstring muscles) and extension (quadriceps muscles) were evaluated at a speed of 60 degrees per second.[147] There were 2 sets of 6 repetitions, and resting interval of 3 minutes between sets. For all tests, participants were verbally encouraged to perform as vigorously as possible. Total strength was defined as the sum of peak torque of knee extension and flexion.

3.7 Final Testing

Six months following the start of the intervention period, all subjects repeated the same testing procedures performed at baseline. This included anthropometric measures, vascular ultrasound measurements, physical function assessment, and blood draws. All testing was conducted by study personnel blinded to the subject's group assignment. Several factors, including blood pressure, heart rate, circulating hormones, and vascular smooth muscle reactivity,

have been implicated in the circadian pattern of cardiovascular events[148]. Thus, each testing and measurement session described above occurred approximately at the same time of day within a 2-hour window as the baseline testing to ensure conformity between baseline and final testing measures.

3.8 Data Management, Statistical Analysis and Power Estimates

3.8.1 Data Management

Data management and quality control was managed using a Microsoft Excel spreadsheet. All data that were not downloaded directly, such as data from serum assays, were entered and double checked for accuracy using duplicate entry forms and compare features programmed in Microsoft Excel. Confidentiality of data was maintained by using only subject identification numbers for data entry. The master document linking participant names and identification numbers and group assignment were maintained in a separate password-protected file on a secure network accessible only by the mentor (Dr. Wilund); therefore, access to the data was restricted.

3.8.2 Statistical Analysis

All statistical analysis was performed using SPSS software version 19.0 (IBM Corporation, Armonk, NY) and significance was based on a two-tailed alpha value of 0.05. Each participant was randomly assigned to one of the two groups (CON, POM) with equal probability.

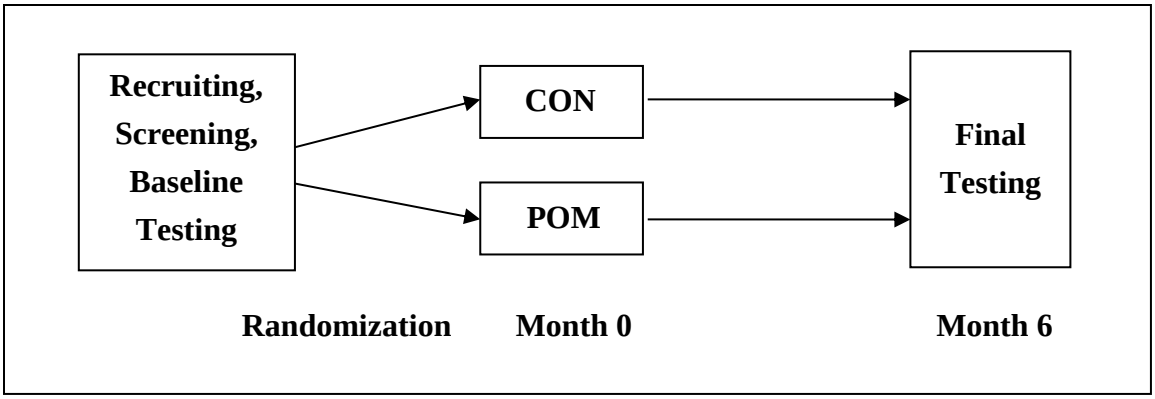
Compliance was defined as ingestion of at least 75% of the prescribed POM or placebo supplements. The data were screened for the presence of outliers in each of the two treatment groups. This was accomplished by using box plots and frequency distributions.

The general analytic framework for the statistical analysis was a Group*Time mixed model ANOVA. The model included the subject as a random effect, and the fixed effects include Group as the main effect, and Group*Time as a 2-way interaction. Group (CON or POM) was used as a between-subjects factor, and Time (baseline and 6 months) as a within-subjects factor. A Chi-square test was used to analyze differences in gender, diabetic and smoking status between groups. Correlation analysis was also performed to assess the relationship between selected variables of interest.

Figure Legends

Figure 2 Study timeline. Subjects were recruited on a rolling basis and randomly assigned to one of the two groups (CON or POM). Patients completed all testing measures at baseline and 6 months after starting the supplementation period.

Figure 2 Study timeline.



CHAPTER 4

RESULTS

4.1 Characteristics of Study Subjects

Thirty-three of the 75 patients that were recruited agreed to participate in the study. These patients were randomly assigned to 2 groups to receive either pomegranate extracts (POM) or placebo (CON). Among the 33 enrolled subjects, 6 (POM: N=3; CON: N=3) dropped out of the study, including 2 that moved to other cities, 1 that received a kidney transplant, 1 that refused to complete the 6-month testing, and 2 that were unable to finish the study due to personal issues. The dropout rate was 19% for the POM group, compared to 18% for the CON group (Figure 3). Adverse events such as stomach upset or other GI-related effects were not observed among subjects. Twenty-seven subjects finished the study, and their 6-month compliance to the study intervention (% of pills consumed) was 95.9±1.0% and 98.2±0.6% in POM and CON, respectively ($p = 0.07$).

As shown in Table 1, general patient characteristics in the POM and CON groups were similar at baseline. There were no significant differences between groups in gender, diabetes or

smoking status. However, baseline systolic and diastolic blood pressures were both significantly higher in POM, compared to CON.

4.2 Cardiovascular Outcomes

There was a significant interaction between group and time for systolic and diastolic blood pressure, as they decreased by 12.8±0.1% ($F_{1, 25} = 6.00$; $p < 0.05$) and 11.2±0.1% ($F_{1, 25} = 7.80$; $p < 0.05$), respectively, in POM, but did not change in CON (Figure 4). However, the changes in blood pressure in the POM group were no longer significant after controlling for subject's baseline blood pressure. There were no interactive or main effects of group and time on heart rate, CIMT, PWV, β-stiffness, augmentation index, stroke volume, cardiac output, or systemic vascular resistance (Table 2).

4.3 Physical Performance Outcomes

There were no interactive or main effects of activity group and time on gait speed, shuttle walk time, functional fitness tests, or muscle strength (Table 2).

4.4 Markers of Inflammation and Oxidative Stress

There were no interactive or main effects of activity group and time on CRP, IL-6 8-OHdG, AOPP, ox-LDL, ORAC, arylesterase, or paraoxonase (Table 2). There was a significant interaction between group and time for lactonase activity, as it increased by 5.4±1.7 kU/L, or 26.6%, ($F_{1, 25} = 4.58$, $p < 0.05$) in POM, compared to no change in CON .

The change in total cholesterol was positively correlated with the change of ox-LDL (r =0.49, p = 0.01) and the change in AOPP (r = 0.42, p = 0.03). The change in ox-LDL was inversely correlated with the change in ORAC (r = -.38, p = 0.04), and positively correlated with the change in 8-OHdG (r = 0.50, p = 0.01). No other significant correlations between major outcome variables were found.

Figure Legends

Figure 3 Study flowchart. Thirty-three patients were recruited and randomly assigned to either pomegranate extracts (POM) or placebo (CON). Six patients (POM: N=3; CON: N=3) dropped out of the study. The dropout rate was 19% for the POM group, compared to 18% for the CON group. Twenty-seven subjects finished the 6-month intervention.

Figure 4 Blood pressure changes from baseline to 6 months. Patients in both the POM and CON groups had blood pressure measured at baseline and 6-month testing. SBP and DBP both decreased from baseline to final testing in the POM group ($p< 0.05$), but did not change in the CON group. 0M and 6M indicate baseline and 6-month testing time points, respectively. Values are given as mean±S.E. Mean; Data was analyzed by 2-way ANOVA. * indicates $p<0.05$ for group x time interaction effect. # indicates $p < 0.05$ for baseline levels between groups.

Figure 3 Study flowchart.

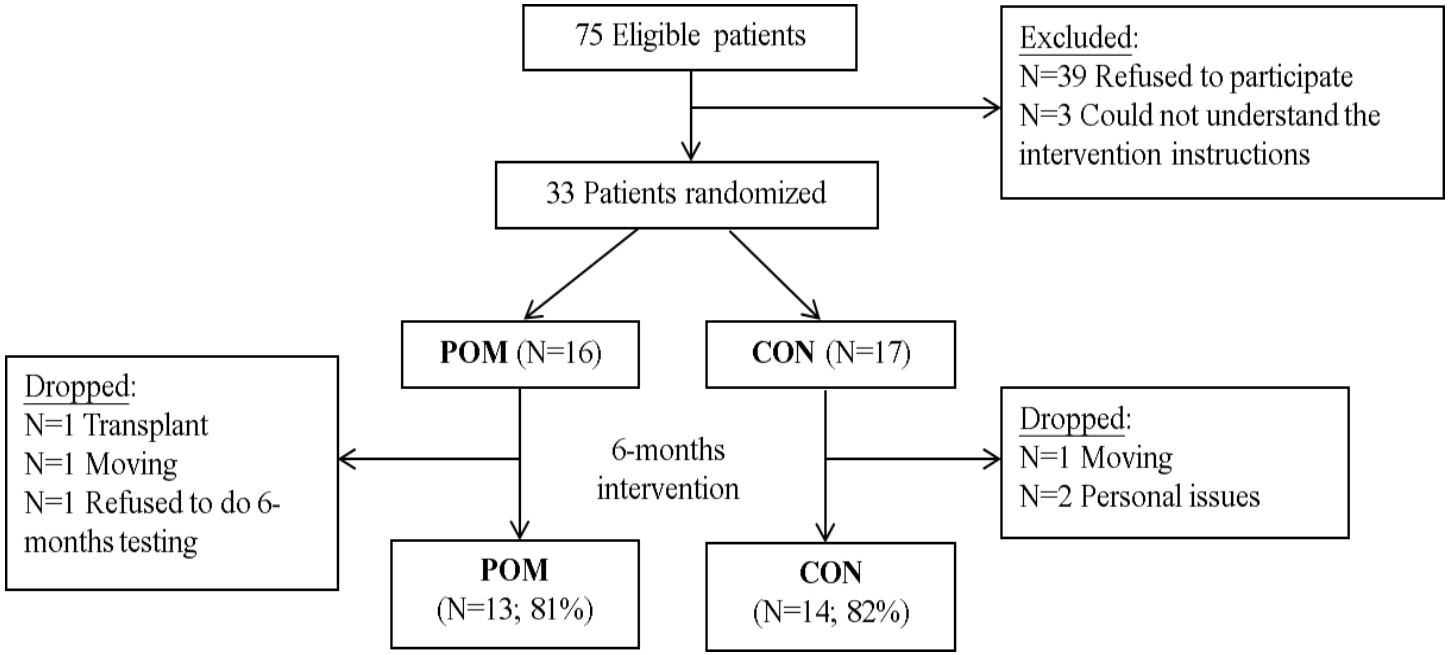


Figure 4 Blood pressure changes from baseline to 6 months.

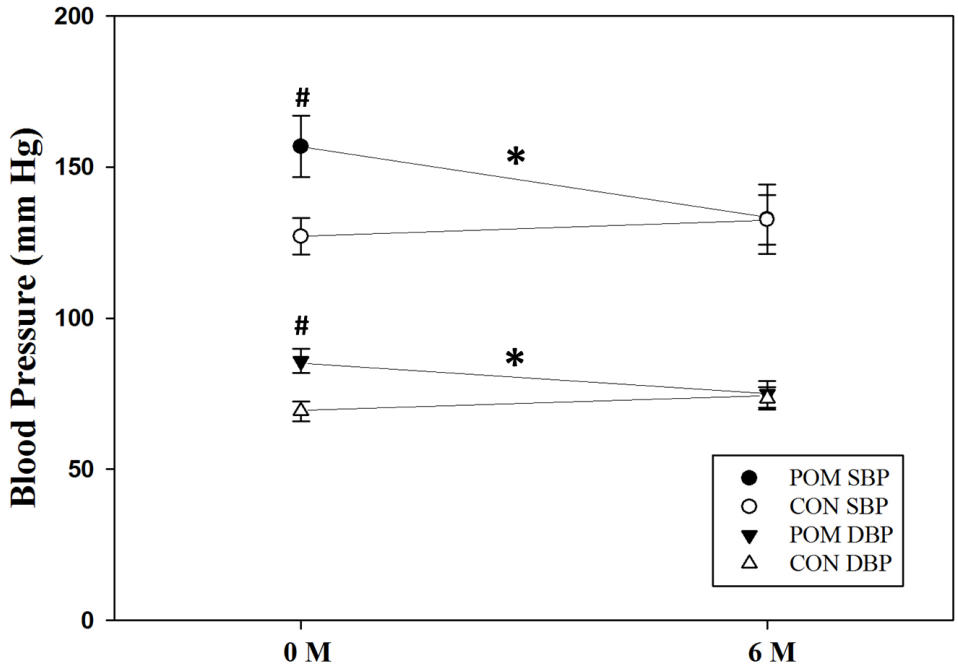


Table 1 Patient characteristics at baseline.

Characteristic	POM (N=13)	CON (N=14)	p value
Age (years)	52.6±3.3	55.9±2.6	.45
Gender (Male %)	61.5	64.3	.88
Ethnicity			.51
Caucasian (%)	15.4	7.1	
African American (%)	84.6	85.8	
Hispanic or Latino (%)	0	7.1	
Diabetes mellitus (Yes %)	53.8	35.7	.34
Smoking status (Yes %)	30.8	28.6	.91
Vintage (months)	75.5±14.1	59.8±10.6	.39
Etiology of CKD			.51
Uncertain (N)	4	2	
Hypertension (N)	6	10	
Diabetes (N)	2	2	
Polycycstic Kidney Disease (N)	1	0	
Chronic Glomerulonephritis (N)	0	0	
Chronic Pyelonephritis (N)	0	0	

Table 2 Outcome variables at baseline and final testing.

Outcomes	POM (N=13)			CON (N=14)		
	N	0M	6M	N	0M	6M
Weight (kg)	12	96.6±8.0	89.9±6.4	14	87.7±8.0	88.7±7.6
Height (cm)	11	167.3±2.9	167.0±2.7	14	167.3±2.1	167.4±2.0
BMI (kg/m^2)	11	32.3±2.1	31.7±2.1	14	31.1±2.5	31.5±2.4
Body fat percentage (%)	11	29.1±3.1	28.9±3.1	13	29.6±2.6	29.5±2.5
Waist circumference (cm)	13	110.0±5.7	107.3±5.1	14	105.7±6.5	106.9±6.5
Albumin (g/dl)	13	4.0±0.1	4.0±0.1	14	3.9±0.1	4.0±0.1
Kt/V	6	1.6±0.1	1.6±0.1	11	1.6±0.1	1.6±0.1
Cardiovascular outcomes						
SBP (mmHg)	13	156.8±10.1#	132.7±11.5*	14	127.1±6.1	132.6±8.2
DBP (mmHg)	13	85.8±4.0#	74.8±4.4*	14	69.2±3.3	73.5±3.7
Mean arterial pressure (mmHg)	13	109.3±5.6#	96.9±5.7*	14	88.4±3.8	93.4±4.6
Heart rate (beats/min)	13	72.9±3.3	68.0±8.2	14	69.3±3.6	68.6±2.9
PWV (m/s)	13	10.2±1.2	11.5±1.6	11	8.6±0.6	10.4±1.4
CIMT (mm)	12	0.54±0.10	0.76±0.04	14	0.70±0.10	0.74±0.06
β-stiffness	12	12.0±2.1	11.3±1.7	12	10.4±1.7	11.6±1.6
Augmentation index (%)	10	32.0±2.1	34.1±5.6	14	23.6±2.2	21.1±3.6
Stroke volume (ml/beat)	10	52.5±4.4	57.5±6.0	12	53.9±4.0	51.0±6.2
Cardiac output (L/min)	10	3.9±0.4	4.3±0.4	12	3.4±0.2	3.4±0.4
Systemic vascular resistance (mmHg/L/min)	10	30.1±3.6	24.7±2.4	12	27.5±2.2	33.6±5.8

Table 2 (cont.)

Outcomes	**POM (N=13)**			**CON (N=14)**		
Physical performance outcomes	**N**	**0M**	**6M**	**N**	**0M**	**6M**
Walk speed (sec)	12	12.4±1.1	13.4±1.2	14	12.0±1.2	11.7±0.9
Shuttle walk time (sec)	12	234.8±44.5	207.8±40.8	14	268.3±33.3	262.7±31.1
Peak torque RKF (ft*lb)	12	39.2±4.7	43.5±7.7	14	34.8±3.7	36.0±5.4
Peak torque RKE (ft*lb)	12	67.6±8.7	75.6±11.4	14	64.5±6.6	76.3±10.6
Peak torque LKF (ft*lb)	12	37.3±5.7	40.9±5.8	14	31.4±4.1	37.0±5.3
Peak torque LKE (ft*lb)	12	69.7±9.8	70.0±9.0	14	65.8±7.9	79.6±12.1
Chair stand (repetitions)	12	11.3±2.4	9.5±1.9	13	10.5±1.2	10.2±1.1
Arm curl (repetitions)	12	14.4±2.3	13.7±2.6	14	14.6±1.3	14.4±1.1
Sit and reach (inches)	12	-4.1±1.0	-3.3±0.9	13	-2.9±1.4	-2.5±1.3
Back scratch (inches)	13	-5.8±1.7	-4.6±1.3	14	-4.9±1.3	-5.7±1.2
8-foot up and go (seconds)	12	9.4±1.6	10.3±2.2	14	8.3±1.4	7.6±0.8

Table 2 (cont.)

Outcomes	**POM (N=13)**			**CON (N=14)**		
Plasma and serum variables	**N**	**0M**	**6M**	**N**	**0M**	**6M**
Total cholesterol (mg/dl)	13	177.7±13.3	186.0±18.5	14	149.6±11.5	157.3±17.1
HDL (mg/dl)	12	48.8±4.9	47.6±4.6	14	49.7±4.5	47.1±4.3
LDL (mg/dl)	12	103.3±13.2	117.5±18.2	14	83.0±12.3	92.0±16.8
Triglyceride (mg/dl)	13	128.2±32.9	104.3±22.2	14	84.8±30.4	91.1±20.5
ORAC (uM)	13	17933.2±2907.0	16586.9±1827.7	14	18100.5±1530.6	17415.0±1391.3
AOPP (uM)	13	163.6±19.9	144.4±10.8	14	165.1±10.5	141.3±13.2
8-OHdG (ng/ml)	13	34.3±3.2	33.1±3.0	14	31.4±3.9	31.5±2.9
Ox-LDL (U/L)	13	34.4±5.1	34.1±6.5	14	31.4±3.9	27.8±4.4
Arylesterase (kU/L)	13	36.1±2.4	37.0±3.5	14	36.0±3.0	41.8±3.2
Lactonase (kU/L)	13	20.3±2.1	25.7±2.4*	14	25.1±2.1	25.5±2.5
Paraoxonase (U/L)	13	707.3±117.0	746.5±127.7	14	741.2±121.3	796.1±130.0
IL-6 (pg/ml)	13	7.5±2.1	7.6±1.7	14	7.6±1.9	7.4±1.4
CRP (mg/L)	13	11.6±4.4	14.4±4.8	14	5.7±2.3	4.6±1.5

0M and 6M indicate baseline and 6-month testing time point, respectively. Kt/V, K represents dialyzer clearance of urea, t represents dialysis time, and V represents volume of distribution of urea. SBP, systolic blood pressure; DBP, diastolic blood pressure; PWV, pulse wave velocity; CIMT, carotid intima-media thickness; RKF, right knee flexion; RKE, right knee extension; LKF, left knee flexion; LKE, left knee extension; ORAC, oxygen radical absorbance capacity; AOPP, advanced oxidation products of proteins; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; ox-LDL, oxidized low-density lipoprotein; IL-6, interleukin 6; CRP, c-reactive protein; paraoxonase, PON-1 activity towards paraoxon; arylesterase, PON-1 activity towards phenyl acetate; Lactonase, PON-1 and PON-3 activity towards dihydrocoumarin. PON-1 activities are expressed as μ moles of hydrolyzed substrate per minute per liter of serum (U/L) or mmol/min/liter (kU/L) as indicated. Values are reported as mean±S.E. Mean; Data was analyzed by 2-way ANOVA. * indicates $p<0.05$ for group x time interaction effect. Baseline data was analyzed by Student's *t*-test or Pearson Chi-Square test. # indicates $p < 0.05$ for baseline levels between groups.

CHAPTER 5

DISCUSSION

The present study is the first study to investigate the effect of pomegranate extract on cardiovascular health and physical function in patients with chronic kidney failure. Our primary finding was that both systolic and diastolic blood pressure was significantly reduced in patients after 6 months of pomegranate supplementation. However, because baseline blood pressures were higher in the POM group, it cannot be ruled out that these changes may have been due to a regression to the mean. Pomegranate supplementation had no effect on any other metrics of cardiovascular risk or physical function in our patients. Taken together, this data suggests that pomegranate extract supplementation may provide limited benefits in terms of reducing the development or progression of co-morbidities in patients with chronic renal failure.

Oxidative stress is believed to affect blood pressure through a variety of mechanisms[149, 150]. Increased oxidative stress contributes to vascular dysfunction by reducing the bioavailability of nitric oxide (NO), which is a major vasodilator (reviewed in[151]). Oxidation of L-arginine and tetrahydrobiopterin (BH4), which are two essential cofactors for endothelial NO synthase (eNOS), results in the decreased formation of NO. In addition, oxidative stress in vascular smooth

muscle cells (VSMCs) can cause cell proliferation or apoptosis, and further contributes to changes in blood vessel diameter and blood pressure[152]. Antioxidants have been shown to improve vascular dysfunction induced by excessive oxidative stress[151]. Antioxidant supplementation, such as vitamin C or E, has shown to protect against endothelial damage by upregulating eNOs and scavenging reactive oxygen species[153]. It is well-established that polyphenolic antioxidants, such as red wine and pomegranate, can function as ROS scavengers, enhance the production and bioavailability of NO, thereby improving vascular resistance and possibly may respond to changes in blood pressure[16, 105, 154-157]. Though not statistically significant, there was a trend for a reduction in TPR in the POM group ($p = 0.11$ for group x time interaction), suggesting that changes in vascular resistance may have partially contributed to the BP reduction in POM. In addition, several studies have demonstrated reductions in blood pressure following pomegranate *juice* consumption in hypertensive subjects and in patients with carotid artery stenosis[16, 158, 159], so there is precedent for the blood pressure reductions that we found following pomegranate *extract* supplementation in our patients. This also suggests that the blood pressure changes we found may not have been solely due to a regression to the mean. Aviram et al. identified a potential mechanism for the blood pressure lowering effect of pomegranate, suggesting that it directly interacts with serum angiotensin converting enzyme (ACE). They incubated human serum with different concentrations of pomegranate juice, and found a pomegranate juice

dose-dependent inhibitory effect on serum ACE activity[159]. However, they found no correlation between the reduction in ACE activity and BP, suggesting the reduction in ACE activity may not be the major factor contributing to pomegranate-induced BP changes.

Research also suggests that pomegranate provides additional cardiovascular benefits besides reducing blood pressure. CIMT has been used as a validated surrogate end-point for atherosclerosis and vascular disease risk[19, 160]. Aviram et al. found a 30% reduction in CIMT among subjects with severe carotid artery stenosis after 1 year of pomegranate juice consumption[16]. Pomegranate juice was also reported to decrease the CIMT progression rates in subjects at moderate risk for coronary heart disease[123], and improved a composite score of CIMT in hemodialysis patients[19]. Despite these previous studies showing beneficial effects of pomegranate juice on CIMT in clinical populations, the present study did not find CIMT improvements with 6 months of pomegranate extract consumption. This discrepancy in the CIMT findings might be due to different forms of supplementation (i.e., powder extracts versus juice), as well as a shorter period of intervention (i.e., 6 months versus more than 1 year) in the present study. Although Aviram et al.[16] found a reduction in CIMT as soon as 3 months of pomegranate juice consumption in subjects with severe carotid stenosis, our dialysis patients had relatively thinner CIMT at baseline (0.54mm CIMT in our study versus 1.52mm CIMT in

Aviram et al.), which might be another potential reason that we did not see changes in CIMT after 6 months of pomegranate extract supplementation.

Our findings are contrary to prior studies suggesting antioxidant supplementation, such as vitamin C and/or E, would reduce vascular disease risk by decreasing arterial stiffness in subjects with diabetes or hypertension[161, 162]. However, Dohadwala et al. showed that PWV did not change after a 4-week intervention with cranberry juice, which contains abundant polyphenolic compounds, in patients with coronary artery disease[163]. Although there is little evidence of reducing arterial stiffness by pomegranate supplements in clinical populations, consumption of pomegranate juice for 4 weeks had no effect on PWV in healthy subjects[164]. Longer clinical trials are needed to investigate the effects of polyphenolic compounds on arterial stiffness to confirm these findings.

Although studies have shown that low plasma antioxidant levels (e.g., tocopherol, carotenoids) are associated with poor skeletal muscle strength and impaired physical performance in older adults[112, 165, 166], to our knowledge, only a few studies have directly investigated the effects of antioxidant supplementation on these metrics. A recent study by Hauer et al. found that N-acetylcysteine (NAC) combined with resistance exercise training improved muscle strength in the elderly after 13 weeks[13]. However, these data must be interpreted with caution because plasma antioxidant levels were not reported to confirm the relationship between

changes of antioxidant level and improved muscle performance. In addition, the improvements in physical performance and muscular strength were not only observed in the group with NAC supplementation combined with resistance exercise, but also in the group with placebo combined with resistance training. These results suggested the functional improvements may be due to resistance training response or learning effects, instead of antioxidant benefits solely.

Pomegranate juice has been used as an antioxidant supplement to improve recovery of elbow flexor strength, as well as to attenuate soreness, after a single damaging bout of eccentric exercise in healthy subjects[14, 15]. However, our data did not show that pomegranate supplementation chronically improves physical function or muscle strength in dialysis patients. This inconsistency may be due to the different populations and functional measures that were assessed, or the fact that we assessed chronic changes in function, as opposed to recovery from a single bout of exercise.

Paraoxonase-1 (PON1) is the most potent HDL-associated antioxidant enzyme, and uremic toxins may play a mechanistic role in PON1 inactivation[167]. Past studies have reported widely variable PON-1 activity in hemodialysis patients[137, 168-170], which may be explained in part by differences in subject's BMI, nutritional status, CRP levels, vintages of hemodialysis, or differences in assay methods used in each study[170]. However, we did not find any relationship between PON-1 activity and patient's BMI, dialysis vintages, albumin or CRP levels.

Pomegranate juice has shown to increase the paraoxonase, arylesterase and lactonase activities of PON-1[123, 131]. PON-1 hydrolyzes organophosphate compounds (e.g., paraoxon) and aromatic carboxylic acid esters (e.g., phenyl acetate), and possesses peroxidase-like activity that can contribute to its protective effect against lipoprotein oxidation[135, 171]. PON-1 also provides its anti-atherogenic properties through a homocysteine-thiolactonase activity[134, 172]. Among arylesterase, paraoxonase and lactonase, we only found an increase in lactonase activity in the POM group. Although these results differ from some published studies showing that PON-1 activity toward other substrates improved after pomegranate juice consumption[124, 136], they are consistent with the recently accumulating evidence suggesting that the lactonase activity is the most significant explanation for PON-1's atheroprotective properties[137, 173-175].

PON-1 activity largely depends on its association with HDL. However, we did not find an association between the change in HDL cholesterol concentration and change of PON-1 activities in the present study, suggesting that the change in lactonase activity is not related solely to changes in HDL concentration in hemodialysis patients. This finding is in agreement with those by Gugliucci et al., which showed no correlation between lactonase activity and HDL levels in CKD patients, and might be due to the inhibition of enzyme catalysis and synthesis by uremic toxins[137].

Punicalagin, an ellagitannin found in pomegranate, accounts for 70-89% of the antioxidant activity of pomegranate juice[116, 176], and studies have shown that pomegranate juice and extracts have similar bioavailabilities. Our 1,000-mg pomegranate extract contains approximately 755 mg of gallic acid equivalents[177]. This is similar to a typical 8-ounce serving of pomegranate juice, which contains 857 mg of gallic acid equivalents. As a result, we chose to use the pomegranate extract in this study, as opposed to the juice, due to the high content of potassium in pomegranate juice, and restrictions on the intake of these minerals in dialysis patients.

Despite very similar antioxidant contents in juice and extracts, prior work on ellagitannin absorption yielded variable results. Seeram et al. showed that ellagic acid concentration in plasma reached its peak concentration 2-3 hours after consuming pomegranate extract, compared to a peak plasma ellagic acid concentration at 1 hour following consumption of pomegranate juice[177]. By contrast, Cerda et al. found that neither punicalagin nor ellagic acid were detected in plasma after consuming 1 liter of pomegranate juice per day for 5 days[178]. The different absorption rates between pomegranate juice and extracts may contribute to the inconsistent findings from the current study, compared to the previous work published by Shema-Didi et al.[19], who reported that pomegranate juice provided anti-inflammatory and cardioprotective benefits in hemodialysis patients.

A potential explanation for why we observed no POM-induced change in circulating markers of inflammation or oxidative stress in the present study may be due to the abnormal distribution of microflora in hemodialysis patients. Cerda et al. suggested that the poor absorption of ellagic acid was explained by its poor solubility at physiological pH in the intestine, and its poor ability to bind to intestinal epithelium[179, 180]. Cerda also suggested that the cardiovascular protective effects of pomegranate might be due to metabolites produced from pomegranate digestion by intestinal microflora, such as urolithins, rather than to the polyphenolic concentration present in the pomegranate products[178]. Excessive uremic toxins, CKD-related comorbidities, medications, and irregular dietary patterns, which are all conditions commonly seen in hemodialysis patients, may cause an abnormal distribution of microflora in the gastrointestinal tract and change of gastrointestinal permeability[181]. Indeed, we found no difference in ORAC, a measure of serum total antioxidant capacity, between groups at 1.5 and 3 hours after pomegranate extract or placebo ingestion (data not shown). The abnormal distribution of microflora in CKD patients might result in a reduction in antioxidant absorption, thereby attenuating the potential antioxidant, and anti-inflammatory effects of pomegranate in hemodialysis patients. Further research is needed to investigate the concentration of ellagic acid in plasma after ingesting pomegranate products to determine the absorption rate of antioxidant in patients undergoing hemodialysis treatment.

Limitations

There are several limitations to our study. Primarily, the sample size was relatively limited, thereby reducing the statistical power for the analysis. This also inhibited us from controlling for subjects' medication status and intercurrent illness, both of which may have affected our primary outcomes. In addition, we did not measure plasma levels of ellagic acid to confirm whether the subjects were actually taking pills during the intervention.

CHAPTER 6

CONCLUSIONS

Chronic kidney disease patients undergoing hemodialysis therapy suffer from a variety of co-morbidities that may be mechanistically linked, such as reduced exercise capacity, increased cardiovascular risk, and elevated inflammation and oxidative stress. These co-morbidities not only contribute to the low quality of life, but also increase the mortality rate in this population. Because current pharmacological therapies have failed to significantly reduce uremic-related co-morbidities in hemodialysis patients, the present study was designed to determine the effects of pomegranate supplementation, a rich source of polyphenol antioxidants, on CVD risk, physical performance, inflammation and oxidative stress. The present study suggests modest benefits of 6-months pomegranate extract supplementation in patients undergoing hemodialysis, including (1) possible reductions in blood pressure, and (2) an increase in serum antioxidant activity. However, pomegranate extract supplementation had no effect on other markers of cardiovascular risk, or muscle strength and function. Though past studies demonstrated relatively robust cardioprotective effects of pomegranate juice consumption in various clinical populations,

including hemodialysis patients[16, 19, 123, 125, 158, 159], our data indicates that the benefits of pomegranate extract supplementation may be limited in hemodialysis patients.

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