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Reestablishing a New Normal: Addressing the Social Injustices at the Heart of Cardiovascular Disease Epidemic and Examining the Role of Feutin-A as a Potential Therapy

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Table of Contents

I. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the world [1]. Nearly 1 in 3 deaths can be attributed to cardiovascular related diseases[2]. Similar to other chronic diseases, CVD disproportionally impacts racial minorities, low-income individuals, and rural populations [3]–[5]. In light of these trends, a thorough examination of the systemic causes of these disparities needs to be performed to better understand how to decrease CVD morbidities and mortalities. Further, these vulnerable populations are more likely to be placed on dialysis when diagnosed with chronic kidney disease (CKD) which expedites the development of CVD [6]. More specific to CVD's cellular pathology, a major contributor to CVD is a condition known as vascular calcification, the ectopic accumulation of hydroxyapatite - the primary mineral that makes up bone – within and on the arterial walls[7], [8]. Once thought to be a passive process of calcium and phosphate deposition within arteries, vascular calcification is now known to be an active, cell-regulated condition[9]. In a calcifying environment, vascular smooth muscle cells (VSMCs) undergo a phenotypic switch to osteoblast like cells that produce hydroxyapatite. At physiologically normal serum levels, the circulating glycoprotein alpha 2-Heremans-Schmind, better known as fetuin-A, is shown to be a potent inhibitor of vascular calcification by clearing free calcium and phosphate from the cardiovascular system[10], [11]. As expected, studies suggest that patients with lower serum levels of fetuin-A experience greater ectopic calcification within their arteries compared to healthy patients[6], [12]–[14]. Interestingly, recent literature suggests that fetuin-A's role as a moderator of calcification may depend on fetuin-A's absorption into VSMCs[11], [15]. This thesis will investigate fetuin-A's absorption into VSMCs and suggest reestablishing fetuin-A levels as a novel means to inhibit vascular calcification –

particularly in CKD patients. Furthermore, the role policy and systemic injustices play in the pathology of CVD, especially vascular calcification, will be discussed.

II. Social Determinates of Cardiovascular Disease

2.1 Overview

Longstanding, systemic forms of racial and economic injustices in the United States have exacerbated the spread of CVD. Black Americans are 30% more likely to die from CVD than non-Hispanic White Americans [5]. Making matters worse, low income individuals are two times more likely to develop CVD when compared to their high income counterparts [16]. These types of nonmedical factors that control the conditions under which people are born, grown, live, work, and age are known as the social determinates of health (SDOH). The SDOH are categorized into five domains: economic stability, education access and quality, health care access and quality, neighborhood and built environment, and social and community context [17]. To better understand the role these areas of the SDOH play in disease development, the various levels at which policy and institutions influence health outcomes needs to be delimited [18]. The framework used to characterize these factors has three stages: upstream, midstream, and downstream [19]. Upstream SDOH are macro-level factors such as public policy created by large instituon and socio-cultural norms. Examples of these are economic, housing, health, and welfare programs along with social constructs such as race, class, and gender [19]. Next lies the midstream factors of SDOH. These occur at the community or individual organizational level and focus on health system concerns such as access, affordability, and utilization. In addition, psychosocial factors like health behaviors are included at this intermediate level, i.e. smoking, physical activity, addiction, and diet [19]. Health delivery systems and behaviors are related to the same level due to the inseparable interactions between how systems drive human behaviors.

Ultimately, downstream effects of the SDOH are micro-level factors that affect individuals. Issues at this level are physiological changes that impact life expectancy and quality of life such as hypertension, blood lipid levels, and hormone releases [19]. Previously, the body of medicine has predominately focused on addressing the downstream factors of the SDOH, essentially pursing research without considering the largescale factors at play [18]. That said, the upstream and midstream factors must be better understood to effectively combat disease such as CVD. In this light, synergy could be found between individual interventions and equity centered policy decisions to make biomedical therapies more effective. Viewing the SDOH through one of the most common sociological frameworks - the life course approach - develops a greater understanding of how upstream and midstream effects impact the downstream outcome that is CVD and vascular calcification [20].

2.3 Life Course Approach to Understanding the Social Determinates of CVD

The life course perspective focuses on how exposure to CVD risk during critical periods of biological development or cumulative exposure to these risk factors leads to health disparities between different social groups [21]. A strong segment of SDOH research describes adverse childhood experiences as a major contributing factor to health outcomes later in life. The socioeconomic and emotional conditions under which infants and toddlers develop becomes essentially embedded in the children's bodies. The following two examples illustrate the importance of early childhood experiences in life-long CVD risk. James et. al found that among high socioeconomic status (SES) adult African American men, those who were of low SES in childhood, younger than 13 years old, were four times more likely to suffer from hypertension than those who were categorized as high SES in childhood [22]. Even men who were lower in SES as adults saw a slightly decreased risk of hypertension (the primary cause of CVD and

CKD) compared to those who improved from low to high SES in adulthood [22]. Researchers in another example showed that early life adversity such as parental unemployment, parental substance abuse, and poor performance in school – problems often created by weak economic conditions - have a statistically significant association with increased serum levels of inflammatory markers (interleikin-6, fibrinogen, E-selectin, sICAM-1) in adulthood [23]. Interestingly, this relationship was only significant in black individuals as white individuals who experienced adverse childhood experiences as well did not have higher serum levels of inflammatory markers, solidifying the idea that additional inequities over the life course contributes to those observed in childhood. Overall, the social conditions under which an individual develops has long term impacts on health. Policy interventions used to decrease CVD must address socio-economic challenges faced by individuals during key phases of development as, oftentimes, adult interventions are too late to prevent the disease.

2.4 Disparities in Chronic Kidney Disease and Vascular Calcification

Black Americans are four times more likely while Hispanic Americans are nearly twice as likely to have kidney failure compared to White Americans [24]. Black patients on average wait a year longer than white patients to receive a kidney transplant and are less likely to survive while waiting for a kidney transplant[24]. As a result, nearly 35% of dialysis patients are Black, although this groups makes up only 14% of the population [24]. Moreover, studies show that rural American of any race are more likely to be placed on dialysis and have less access to regular kidney care to mitigate added CVD risks associated with CKD [25]. Cardiovascular complications are the leading cause of death for patients battling chronic kidney disease. A primary downstream cause of these deaths is the toll treatments like dialysis take on the body, often removing valuable components of the blood that naturally protect individuals against

calcification – particularly the protein fetuin-A [26]. Therefore, it comes as no surprise that populations impacted by CKD are shown to experience the highest rates of vascular calcification [27]–[29]. When reviewing troubling statics as these, the scientific body should ask why these groups are impacted at greater rates, not only the fact that these communities are at greater risk.

2.5 Case Study: Connecting Public Policy to CVD

How public policy amplifies or fails to address pre-existing social injustices is often discussed, but this analysis does not often connect policy directly to physiological changes. As supported by various iterations of the farm bill, the US Government heavily subsides many crops, famously corn [30]. Agricultural subsidies keep costs low for the consumer while providing farmers protection from financial instability [30]. Historically, farmers grow more of the crop that is being subsidized which often leads to a surplus. Over the last few decades, the most subsidized crop has been corn [31]. Due to the surplus of low-cost corn, high fructose corn syrup (a sweetener derived from corn) quickly replaced other natural sweeteners such as cane sugar [31]. Numerous studies have linked increased consumption of high fructose corn syrup to diabetes and obesity: the primary causes of both CKD and CVD [30], [31].Today, high fructose corn syrup is used to sweeten a plethora of highly processed foods from sandwich bread to juice drinks to entire fast-food meals. The rise of this low-cost sweetener has resulted in foods that are much cheaper than their natural alternatives [31]. Considering the systemic social inequities present today that are mediated by both race and wealth, individuals in disadvantaged communities do not practically have many options beside purchasing these cheap, calorie rich, fast foods to survive. For example, in the 1960s, discriminatory lending policies and racially restrictive zoning laws prevented blacks from accessing safe, quality housing [32]. These segregated neighborhoods were appraised at lower values, which reduced amounts of property tax collected

– taxes that are used to fund infrastructure, education, and other institutions that promote economic growth [33]. Furthermore, black Americans were excluded from many of the early social programs of the nineteenth century following centuries of enslavement, only increasing the already large racial wealth gap. In the context of this and many other economic disparities that impact all races, albeit to different degrees, low-cost, subsidized processed foods are often the best quality these individuals can afford. These poor food options in combination with structural factors preventing upward mobility and equal access to health care cumulate in conditions like vascular calcification. Increased subsides for healthier alternatives provides a short-term, politically favorable solution, but ultimately complex policy problems such as the rising wealth gap are difficult to resolve in a short period of time. Until these systemic problems are addressed, society must look toward providing equitable access to treatment options to minimize the damage caused by CVD. In this light, fetuin-A, a known inhibitor of vascular calcification, shows much potential to meet this need.

III. Fetuin-A's Role in Preventing Vascular Calcification

3.1 Background

Vascular smooth muscle cells are derivatives of mesenchymal stem cells; however, VSMCs are not terminally differentiated [28]. As a result, VSMCs can expresses several unique phenotypes as illustrated in Figure 1.

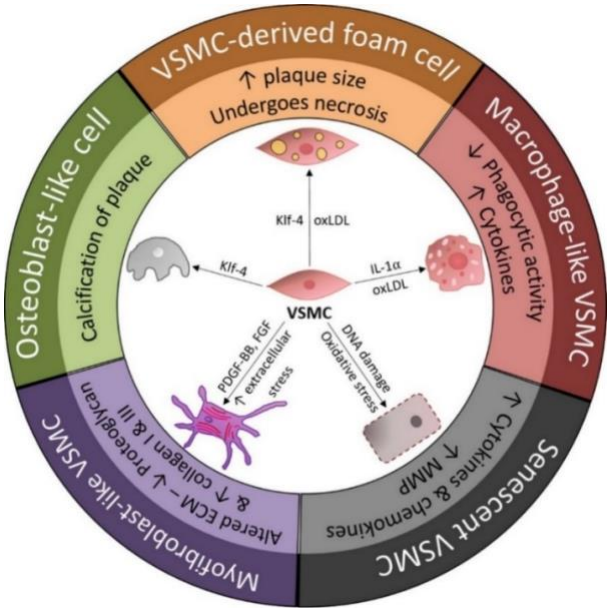

Figure 1. Various phenotypes VSMCs are known to express. Current literature indicates that VSMCs express the same proteins when transdifferentiated to the above cell types as cells who natively express these phenotypes [34].

Vascular calcification is mediated by VSMC's phenotypic switch to osteoblast-like cells. Osteoblasts are responsible for creating the hydroxyapatite scaffold that is responsible for bone's rigid structure [28]. In disease state conditions, VSMC's osteoblast-like phenotype is upregulated resulting in hydroxyapatite plaques forming ectopically within the artery [27]. These phenotypically osteoblast-like VSMCs express much lower levels of alpha-smooth muscle actin which is a key marker for healthy vascular function [28]. Several factors can contribute the

disease state conditions that result in VSMC's phenotypic switch; yet, the most common factors can be grouped into one of three categories: mineral imbalance, inflammation, and mechanical injury [27]. The simplest causes of ectopic calcification are hypercalcemia and hyperphosphatemia, abnormally high levels of calcium and phosphate in the blood respectively. Oftentimes, individuals undergoing hemodialysis as a treatment for CKD are stripped of circulating proteins like fetuin-A that moderate blood mineral levels [26]. Additionally, inflammatory agents such as inflammatory cytokines tumor necrosis factor- alpha, C-reactive protein, and CD40-CD154 are shown to trigger calcification [27]. Likewise, mechanical damage to arteries such as the placement of a stent during angioplasty or high blood pressure can induce this calcifying phenotype [35]. The mechanism of this mechanically induced switch is not well understood but is likely the result of the WNT signaling pathway [28], [35].

3.2 Intimal and Medial Calcification

Vascular calcification is characterized by the location within the arterial wall calcifications are found. When mineral plaques build up in the intimal layer of the artery, this is known as atherosclerosis. These plaques can develop fairly quickly and sometimes cause a build-up of cholesterol or other lipids that can lead to blockages [36]. On the other hand, mineral can also deposit within the medial layer of the artery and cause arterial stiffness which decreases the circulatory system's ability to move blood effectively [36]. This condition is known as Monckeberg sclerosis. Figure 2 provides a visual representation of the intimal and medial calcification.

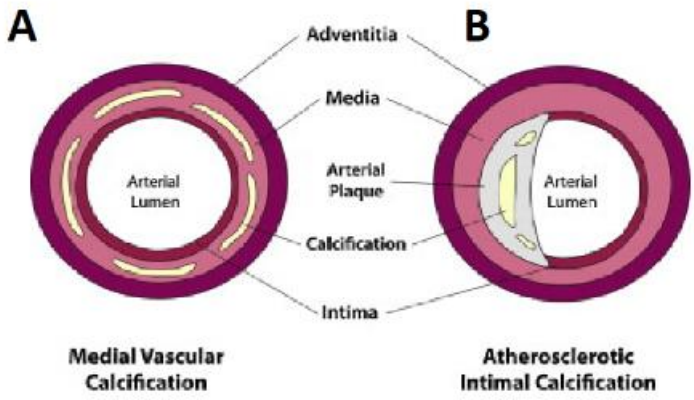

Figure 2. Locations of Calcification A) Medial calcification. B) Intimal calcification [37]

Further, intimal and medial calcification are thought to be distinct physiological processes. In the case of atherosclerosis, serum mediators such as inflammatory agents and serum calcium levels are the primary cause of calcification. As the VSMCs that make up the intima are washed over with these agents in blood, the osteoblast like phenotype is expressed causing plaque buildup inside of the artery [38]. On the other hand, VSMCs located within the media undergo the calcifying phenotypic switch more so due to mechanical stress and systemic diseases like diabetes, lupus, and aging [38]. Regardless of the trigger for the phenotypic switch, both locations of calcification are the result of VSMC induced calcium precipitation.

3.3 Fetuin-A Mediated Calciprotein Formation

Although healthy serum fetuin-A levels are shown to be associated with lower incidences of both medial and intimal calcification in clinical settings, fetuin-A is a particularly potent inhibitor of intimal calcification [11], [38]. This is due to the idea that fetuin-A primary interacts with the intimal arterial lining as blood flows through the circulatory system. Much work has been done to understand how fetuin-A clears excess calcium and phosphate from the blood stream. The consensus is that fetuin-A forms in the liver, gets released into the blood stream then stabilizes

free calcium-phosphate clusters into calciprotein particles (CPPs) that are filtered from the blood by the kidneys and excreted through urine [10], [11]. Current work suggests that fetuin-A primarily prevents calcium phosphate from precipitating as hydroxyapatite; therefore, fetuin-A does not remove precipitated hydroxyapatite from calcified vasculature [11]. Exactly how fetuin-A forms CPPs continues to be investigated, but the most accepted suggestion is that fetuin-A has a negatively charged cystatin domain that attracts positive calcium ions. Cystatin domain 1, labeled green in Figure 3, illustrates the region of fetuin-A hypothesized to be responsible for binding calcium phosphate [11], [41].

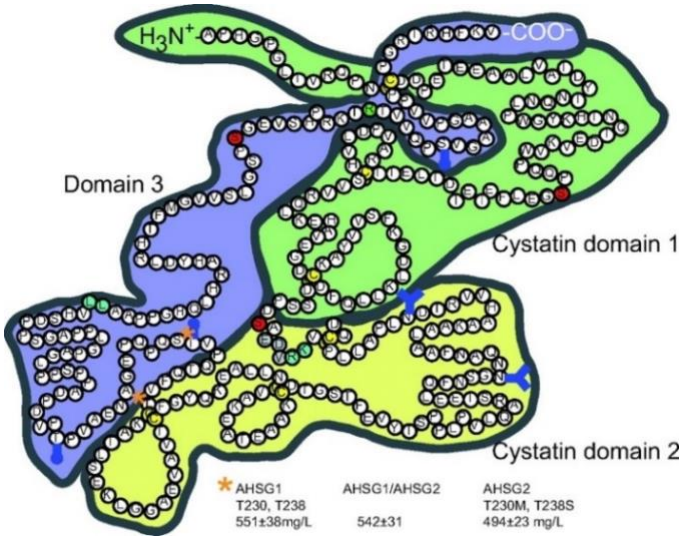

Figure 3. Illustration of human fetuin-A, alpha 2-Heremans-Schmind glycoprotein. Previous work predicts that cystatin domain 1 is responsible fetuin-A's ability to form CPPs [11].

CPP formation is a complex process that goes through many phases to from the most stable particle, simplified in Figure 4. Hydroxyapatite precursor particles of calcium and phosphate, known as Posner clusters - $Ca_9(PO_4)_6$, form in serum under hypercalcemic conditions [11]. Fetuin-A cystatin domain 1's negative charge attracts the largely positive Posner clusters. Several of these protein-mineral complexes associate to form a spherical primary CPP. After more time passes, the primary CPP further stabilizes into a secondary CPP that consist of a

crystalline mineral core with a shell of fetuin-A [11]. The barrier fetuin-A makes around the secondary CPP seen in Figure 4d decreases mineral interaction with serum and the vasculature, resulting in more effective clearing and inhibition of hydroxyapatite precipitation [41].

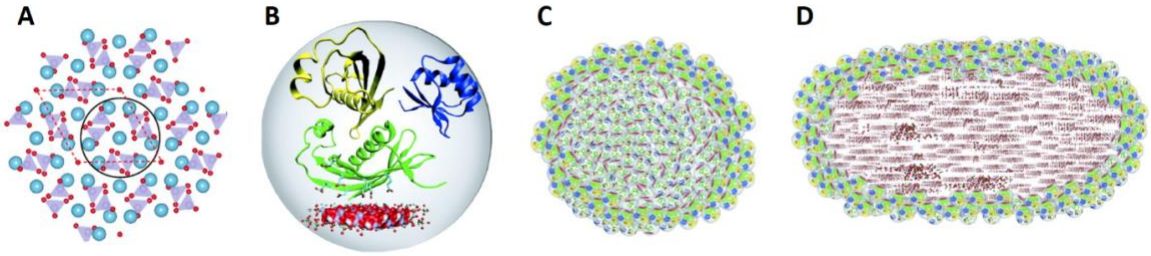

Figure 4. Calciprotein Formation and Stabilization. A) Group of interacting phosphate (blue) and calcium (red) particles that form hydroxyapatite once precipitated. The black circle represents a Posner cluster. B) Homology model of fetuin-A forming a mineral interface at cystatin domain 1(green) with calcium phosphate. C) Primary CPP showing association of fetuin-A/calcium complexes. D) Stabilized secondary CPP showing fetuin-A molecules forming a shell around amorphous calcium phosphate[11].

3.4 Calcifying Matrix Vesicles

Aside from fetuin-A's established role preventing calcification as a circulating glycoprotein, current literature suggest that fetuin-A may play a more important role by preventing mineralization of matrix vesicles (MVs) released from transdifferentiated VSMCs[42]–[44]. Recognizing that vascular calcification is classified as an active, cell mediated process, a greater emphasis is being placed on the role MVs play in propagating mineralization. According to literature, osteoblasts release calcifying MVs to stimulate bone formation[34]. Since VSMCs in their synthetic state display an osteoblast-like phenotype, several experiments have been performed to investigate VSMC's ability to excrete calcifying MVs. Repeatedly, studies have shown that calcifying VSMCs release calcifying MVs, illustrated in Figure 5.

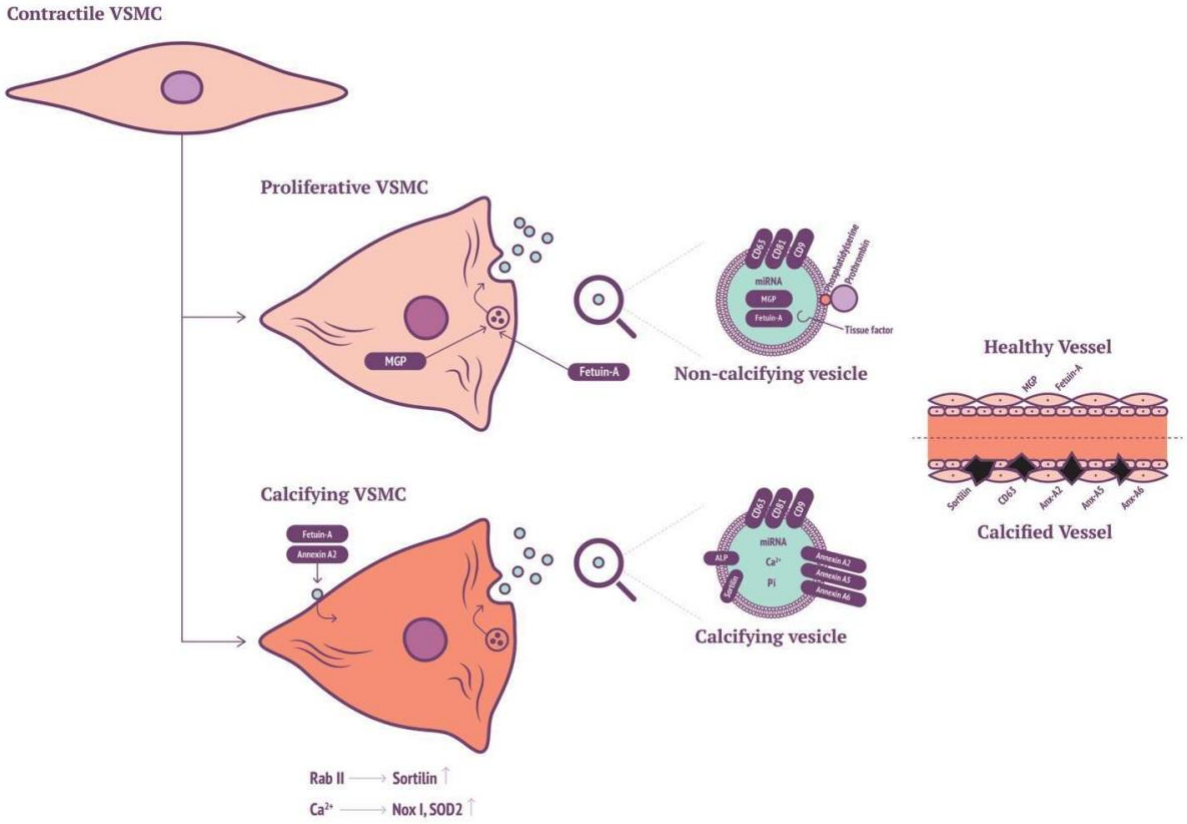

Figure 5. VSMCs in their synthetic, transdifferentiated, state release MVs. If fetuin-A is absorbed by the cell and packaged into these vesicles, calcification is inhibited. If fetuin-A uptake is prevented, these vesicles have been shown to cause calcification[45].

Of note, when fetuin-A is present in MVs secreted by VSMCs in their diseased state, the MVs containing fetuin-A calcify significantly less than those without fetuin-A as described by Figure 6.

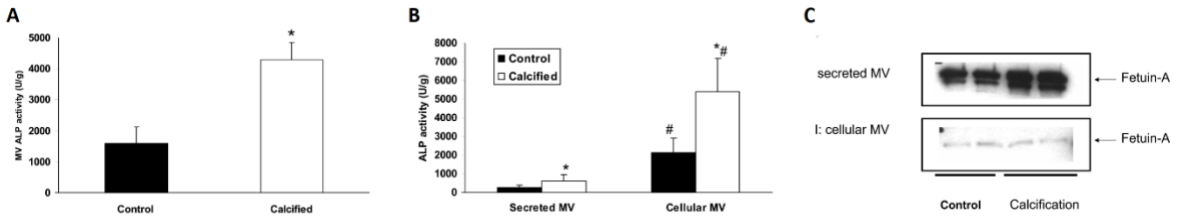

Figure 6. Calcification of Secreted vs Cellular Matrix Vesicles. A) Increased MV ALP activity is shown to be related to the magnitude of calcification observed. B) Secreted MVs are shown to have less APL activity compared to cellular MVs. C) Secreted MVs are shown to contain much

more fetuin-A than cellular MVs. As a result, it is concluded that MVs containing less fetuin-A have increased ALP activity which is related to greater calcification[44].

3.5 Cellular Uptake of Fetuin-A

Based on previous work characterizing fetuin-A being found in vesicles released from VSMCs, fetuin-A must be absorbed by VSMCs, yet little work has been done to characterize fetuin-A's uptake and translocation inside VSMCs. Work by Chen et al. established that fetuin-A uptake is likely mediated by annexin II in VSMCs and dependent on free calcium ions in serum [15]. Annexin II must bind to both calcium and fetuin-A for cellular uptake to occur. Fetuin-A and calcium is absorbed into the cell via pinocytosis of the cell membrane and packaged into a multivesicular body within the cell [43]. Once these multivesicular bodies are released, they are easily cleared from collagen surrounding VSMCs, thus not causing calcification of the vessel.

3.6 Physiologically Normal Serum Fetuin-A Levels Inhibit Calcification in Both Calcifying and Uremic Conditions

Fetuin-A's capability to chaperone free calcium from the blood stream inhibits the calcium phosphate buildups that lead to atherosclerosis. As mentioned in previous sections, CKD patients, especially those receiving hemodialysis, will likely develop atherosclerosis. Two hypotheses explain this phenomenon: 1) hemodialysis reduces serum fetuin-A levels and 2) CKD induces a uremic environment that promotes mineral deposition.

Healthy patient fetuin-A serum concentrations average 15μM while patients undergoing dialysis typically have serum fetuin-A levels below 9μM [39]. This is likely both a physiological response caused by less fetuin-A being produced along with fetuin-A being filtered out of the patient's blood stream by the dialysis process and not being replaced. In support of clinical data suggesting low fetuin-A levels are associated with increased intimal calcification, experimentation shows that fetuin-A concentrations similar to those observed in dialysis patients

is not sufficient to prevent calcium deposition. Conversely, normal concentrations of fetuin-A significantly inhibited calcium deposition, illustrated in Figure 6.

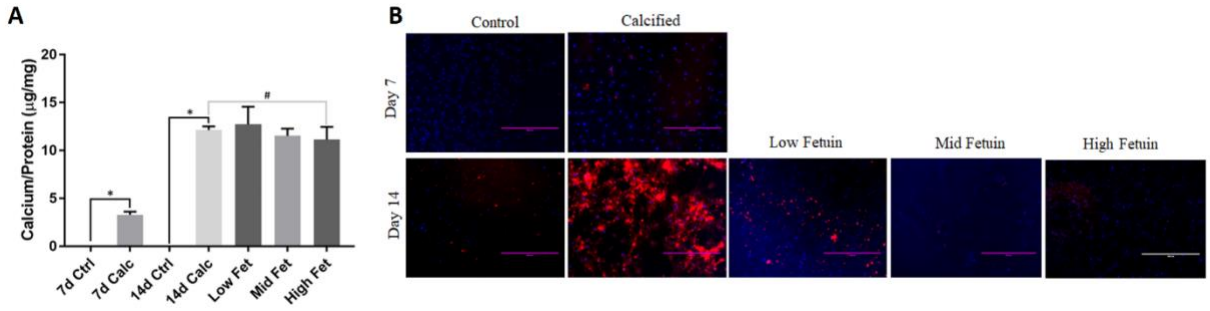

Figure 6. Increased fetuin-A concentrations inhibit calcium content in VSMCs. A) Calcium content is seen to be higher in cells cultured in calcification media than cultured in control media (* denotes $p < 0.05$). Calcium content in cells fed calcifying media treated with high fetuin-A concentration (15 µM) is seen to be significantly lower (# denotes $p < 0.05$) than cells treated with calcifying media only. Cells cultured in calcifying media and treated with low fetuin-A concentration (9 µM) show no significant difference in calcium content as compared to cells cultured in calcification media for the same period of time with no treatment. B) Imaging shows that calcium deposition (orange) is strongly observed at day 14 in calcifying media while calcium deposition is minimal in the high fetuin treatment group.

Furthermore, the loss of renal function observed in late-stage CKD patients is known to cause an increased concentration of uremic toxins in the blood stream [40]. Studies suggest that these toxins induce VSMC calcification through the induction of inflammatory pathways [12]. Notably, indoxyl sulfate (a uremic agent) is shown to accumulate in the serum of CKD patients and cannot be sufficiently removed by means of conventional dialysis [40]. Data in Figure 7 demonstrates that indoxyl sulfate induces calcification of VSMCs in vitro. Additionally, treatment of VSMCs with physiologically normal fetuin-A serum concentrations are shown to inhibit calcification in the uremic environment induced by indoxyl sulfate.

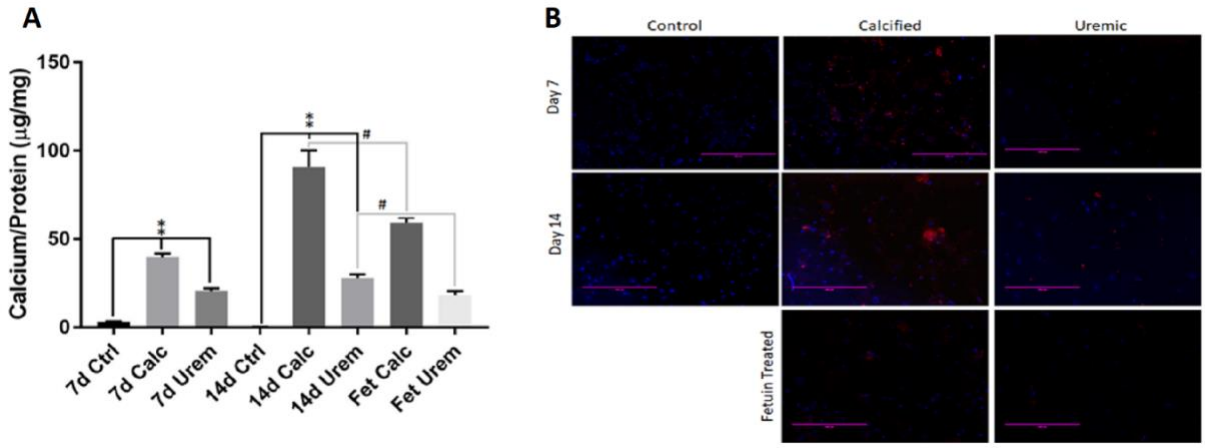

Figure 7. Fetuin-A inhibits calcification in an uremic environment. A) Calcium content is observed to be significantly higher in indoxyl sulfate induced uremia compared to control group ($p < 0.05$). Treatment with 15 µM fetuin-A is shown to significantly reduce calcification in uremic conditions ($p < 0.05$). B) Image shows increased calcification (orange) in uremic conditions while decreased calcification in uremic conditions after treatment with fetuin-A.

IV. Discussion

Well documented systemic injustices plague not only the United States healthcare system, but also the foundations of our nation's largest instituons. Centuries of racial segregation, economic inequality, and poor access to health resources in rural areas have manifest themselves through the CVD epidemic. Making matters worse, current treatment options often fall short in preventing future disease and sometimes induces new problems. While dialysis is a critical treatment to extend the life of those afflicted with late-stage CKD, vascular calcification is a common result due to the uremic environment and removal of natural anti-calcification agents. One of these agents in particular, fetuin-A, is shown to inhibit ectopic calcification. Resulting, this thesis provides clear evidence that reestablishing fetuin-A levels to normal physiological conditions in CKD patients undergoing dialysis inhibits calcification; thus, clinical delivery of fetuin-A has the potential to reduce CVD mortality associated with CKD. However, a new normal must be created to end and remedy the racial and economic injustices at the root of the epidemic, as structural change is how the maximum number of lives will be protected from CVD.

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