THE ASSOCIATION OF SELENIUM LEVELS WITH MARKERS OF CARDIOVASCULAR DISEASE

by

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ABSTRACT

Background: Oxidative stress is a key precursor to atherosclerosis, endothelial dysfunction and arterial stiffness, which are three mechanisms of the progression of cardiovascular disease (CVD). Selenium (Se) is an essential mineral that comprises at least 25 selenoproteins in humans. Many of these selenoproteins play antioxidant roles that are crucial to arterial health and endothelial function. A 2015 meta-analysis of observational studies proposed that CVD risk is significantly decreased only within the narrow selenium range of 55 to 145 µg/L.

Methods: Data were previously collected from the Women and Infant Study of Healthy Hearts (WISH). Serum samples from this study were analyzed to determine Se concentrations using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The distribution of Se in this sample was examined as were linear and quadratic associations with carotid intima-media thickness (cIMT), pulse wave velocity (PWV) and flow mediated dilation (FMD). Sub-group analyses were performed to examine these relationships within and outside of the reported beneficial range of Se status.

Results: Se concentrations ranged from 58 to 598 µg/L, with a median of 172 µg/L. No participants were deficient in Se, but 74% had selenium levels higher than 145 µg/L. The distribution of Se was skewed left, so Se levels were natural log transformed for the analyses. Quadratic relationships between Se level and cIMT, FMD and PWV had better fit compared to

linear relationships. There were no significant associations between Se status and cIMT (p=0.14), PWV (p=0.51) or FMD (p=0.51). There was a significant linear relationship between Se levels greater than 145 µg/L and PWV (p=0.006).

Conclusions: On average, the sample had good cardiovascular health and was relatively young to observe subclinical CVD progression. There may be an inverse association between selenium status and pulse wave velocity, a marker of arterial stiffness. This novel finding may lead to a greater understanding of the mechanisms of arterial stiffness, a major risk factor for CVD, which has great public health importance.

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PREFACE

I would like to thank my committee members for their advisement and support throughout this project. I would also like to thank Dr. Dan Bain at the University of Pittsburgh's Department of Geology and Planetary Science for his time and assistance with the selenium analysis.

1.0 INTRODUCTION

Selenium (Se), which was discovered in 1817, was historically considered a toxic element. In the 1930s, a link was determined between selenium deficiency and cardiomyopathies in livestock animals[1]. Simultaneously, rural areas of China were plagued with endemic cardiomyopathies and osteoarthritis[1]. These diseases were not attributed to Se deficiencies until the 1960s. It is now understood that selenium is an essential trace mineral that is incorporated into the structure of at least 25 proteins in humans. These proteins play integral roles in immune function, reproduction, brain function, thyroid function, metabolism and DNA synthesis[2,3]. Both high and low Se levels have been observed to increase the risk of many adverse events including thyroid diseases, autoimmune diseases, cancers, and cardiovascular disease (CVD)[3].

Selenium plays a role in CVD, at least partly due to the antioxidant activity of numerous selenoproteins. Oxidative stress is a key component in the development and progression of cardiovascular disease[4,5]. Reactive oxygen species (ROS) are produced during the metabolism of oxygen and may be induced by environmental factors. Antioxidants are needed to maintain the reduction-oxidation (redox) balance of the cell[5]. When cells are unable to efficiently clear ROS the redox balance is skewed towards oxidation. Oxidative stress results in the accumulation of oxidized lipids, and the activation of the immune response, inflammation and potentially cell death[6]. Chronic oxidative stress creates a cellular environment susceptible to atherosclerosis, endothelial dysfunction and arterial stiffness[5]. Selenoproteins are responsible for reducing ROS

and preventing oxidative stress in somatic cells throughout the body[2]. However, the balance between oxidant species and antioxidant species must be maintained because critically low levels of ROS can inhibit cell signaling and function[5].

1.1 SELENIUM INTAKE AND METABOLISM

Selenium intake varies widely geographically based on diet, supplementation and soil quality and fertilization. The availability of selenium to plants varies due to soil speciation, pH, organic-matter content, and the presence of other ions[2]. Acidic soil reduces uptake by plants. Selenium can complex with iron and aluminum in the soil, which renders it unavailable to plants[7].

The most common dietary sources of Se include organ meat, seafood, cereals, and grains[2]. Therefore, populations with high consumption of livestock animals and seafood generally have adequate Se intake. On average, males consume more selenium than women[6]. Japan and North America have the highest mean intake of Se with men consuming about 120-140ug/day and women consuming about 80-120ug/day[6,8]. China has the lowest intake of Se at an average of 30-50ug/day[6,8]. In the 1930's an outbreak of cardiomyopathy, called Keshan disease, was discovered in areas of rural China[1]. The etiology was unknown until population studies began in the 1960s which determined selenium deficiency as a likely cause[1]. Due to low Se intake, diseases caused by selenium deficiencies, such as Keshan disease and Kashin-Beck disease, were endemic in China until the early 1990s[1,2,9]. High-selenium crops and Se containing fertilizers are commonly used to increase the selenium content in plants and the livestock animals that consume them.

The intake of selenium is difficult to measure due to the geographic variation of soil and variability in diet trends across cultures. The selenium content of crops varies widely depending

on where they are grown so it is difficult to construct standardized questionnaires that accurately assess intake. Additionally, recall bias and portion size discrepancies prevent nutritional surveys from precisely measuring most micronutrients. Thus, selenium levels are used as a more objective measure of selenium intake[10]. There are many acceptable biospecimens from which to measure selenium status including serum, plasma, whole blood, hair, toenails and urine[3,6,10,11]. Glutathione peroxidase activity and the expression of other selenoproteins have also been used as surrogate measures for selenium status[3]. Serum, plasma and urine are able to reflect short term selenium status, while hair and toenail selenium reflect more long term concentrations[3].

Once ingested, selenocompounds are absorbed primarily through the duodenum[10]. The efficiency of absorption and metabolism depends on the form of selenium ingested. The two main organic forms of selenium are plant based selenomethionine and animal based selenocysteine[2,10]. Selenoproteins are proteins that contain the amino acid selenocysteine. Selenocysteine (Sec) is the 21st amino acid and is coded for by the UGA codon, which is also a stop codon[12,13,14]. Therefore, selenoprotein mRNA contains a selenocysteine insertion sequence and other specialized translational machinery in the 3' untranslated region of the mRNA. This allows translation to continue through the UGA codon[12,13,14].

Selenomethionine can be nonspecifically incorporated into other proteins in place of methionine[9]. However, this form is less efficient in forming selenoproteins as it must first be converted to selenocysteine and further reduced to hydrogen selenide (H_2Se)[9,15]. There are also inorganic sources of selenium which are commonly found in supplements, including selenite and selenate[9]. These inorganic forms are already reduced, so they can be efficiently incorporated into selenoproteins. Because of this efficiency, inorganic forms are more likely to cause toxicity compared to the organic molecules[9, 16].

1.2 ROLES OF SELENOPROTEINS IN CARDIOVASCULAR DISEASE

The current epidemiological evidence on the relationship between selenium and cardiovascular disease is conflicting (Appendix A). Most clinical trials investigating the use of selenium supplementation have shown no improvements in cardiovascular risk factors[17,18,19]. However, the majority of supplementation studies have been conducted in samples with adequate selenium levels.

Inadequate selenium concentrations and selenoprotein expression have been associated with an increased risk for oxidative stress, platelet aggregation and inflammation[2,7,9,20,21]. Two meta-analyses have investigated the relationship between selenium levels and CVD in observational studies and clinical trials. Both of these meta-analyses found that selenium status is significantly associated with decreased incidence of CVD, but the 2015 study found that CVD incidence was significantly reduced only for selenium levels between 55 and 145ug/L[18,19]. Thus, a U-shaped association between selenium concentration and CVD morbidity and mortality may exist[7,9,19].

Selenoproteins are proteins that contain selenocysteine; there are over 25 known selenoproteins in the human body[2,7]. Glutathione peroxidases (GPx) are a family of enzymes with numerous antioxidant functions. There are five isoforms each with selenocysteine at their active center[20]. Three isoforms have been implicated in CVD: GPx1 is found in the cytosol, GPx3 is found in plasma and GPx4 is found in phospholipid membranes[9]. These enzymes function to remove hydrogen peroxide, lipid peroxides, phospholipid hydroperoxides, cholesterol hydroperoxides, superoxides and hydroxides from cells[10,22,23]. By eliminating excess ROS, GPx enzymes play an integral role in regulating oxidative stress within the cell. Due to its antioxidant properties in vascular tissues, GPx1 activity has been determined to be a strong predictor of

cardiovascular disease risk[2,9,22,23]. In a cohort of individuals with coronary artery disease, erythrocyte GPx1 activity was significantly and inversely associated with the incidence of CVD events over a median follow up of 4.7 years[23]. Functional variants of GPx3 have been associated with increased risk for stroke in both adolescents and young adults[23,24]. Decreased expression of GPx3 has been shown to increase the risk of stroke in mice models[25]. GPx4 is located in the membrane of endothelial cells and has been implicated in the reduction of lipid and phospholipid peroxidation within these membranes[26,27].

Selenium is also a component of another family of enzymes responsible for balancing cellular redox processes, the thioredoxin reductases (TrxR). Selenocysteine is present in the C-terminus of TrxR enzymes[28]. This group of enzymes is responsible for regenerating thioredoxins (Trx) in a nicotinamide adenine dinucleotide phosphate (NADPH) dependent mechanism[17]. There are 3 isoforms of the Trx enzyme, which have multiple antioxidant functions and are involved in cell signaling processes[9,28]. TrxR's can directly reduce hydroperoxides, but also play an antioxidant role in the reduction of thioredoxin, which also serves as an antioxidant enzyme[28]. The Trx1/TrxR1 complex has been observed to reduce oxidative stress in cardiomyocytes in vitro[9].

Selenoprotein P (SEPP1) is another important selenoprotein in plasma. Selenocysteine is contained in both the C-terminus and N-terminus of this protein[28]. This selenoprotein is produced by the liver and acts as a transporter to supply extrahepatic tissues with selenium, but also can act as an antioxidant enzyme[28]. There is evidence that SEPP1 inhibits insulin induced production of ROS and the AMP kinase signaling cascade[7]. Other selenoproteins observed to be involved in cardiovascular disease progression are SelR and SelK. SelR is responsible for reducing oxidized methionine on proteins and has been shown to be upregulated during times of cardiac stress in mice models[9]. SelK is located in the membrane of the endoplasmic reticulum and has been shown

to reduce oxidative stress in cardiomyocytes[9]. SelK has also been observed to promote foam cell formation in vitro[9].

1.2.1 Atherosclerosis

Atherosclerosis is a key mechanism in the development of CVD involving chronic inflammation, oxidized lipid accumulation in the vasculature and the formation and hardening of plaques[5]. This is a natural process that occurs with aging, however genetics and lifestyle factors affect the rate at which atherosclerosis progresses. Atherosclerotic progression can be quantified using carotid artery intima media thickness (cIMT).

GPx1 is an antioxidant enzyme located in endothelial cells. A cohort study in 2007 found that GPx1 activity was inversely related to atherosclerosis progression determined by cIMT[29]. Functional variants of GPx1 have been associated with increased cIMT in a study of individuals with Type 2 Diabetes[22]. This indicates that there are genetic components that may interact with selenium status to modulate CVD progression.

GPx3 is an antioxidant enzyme that circulates in plasma[9,34]. This enzyme is able to reduce extracellular hydrogen peroxide and lipid hydroperoxides[34]. It may prevent plasma LDL oxidation[9]. The oxidation of LDL-c in the endothelium is a key risk factor for developing atherosclerosis[30].

A known single nucleotide polymorphism (SNP) in the GPx4 gene alters the enzyme's antioxidant function[31]. Individuals with this SNP have increased lipid oxidation and increased adhesion of monocytes to endothelial cells, which are both important risk factors for atherosclerosis[31]. In mice models, overexpression of GPx4 has been shown to protect against atherosclerosis[5,25].

Interestingly, SelK has also been observed to to promote foam cell formation, which is an early step in the progression of atherosclerosis[9]. In vitro, SelK has been observed to promote the expression of an integral membrane protein in macrophages, which leads to increased uptake of oxidized LDL cholesterol[54].

Studies involving selenium supplementation frequently produce null results. This could be in part due to the reported U-shaped association between selenium status and morbidity. Evidence shows that selenium supplementation does not affect the progression of atherosclerosis[32]. Additionally, no association between toenail selenium status and cIMT or coronary artery calcification (CAC) was found in a cohort of 3112 Americans ranging from 20 to 32 years old[33].

1.2.2 Endothelial Dysfunction

Endothelial dysfunction occurs when there is dysregulation of adhesion molecules, cytokines, chemokines and leukocytes in the endothelium[30]. This dysregulation induces imbalances in signaling between vasoconstriction and vasodilation. Nitric oxide (NO) is an important mediator of endothelium-dependent vasodilation, and also reacts rapidly with ROS. Decreased bioavailability of NO leads to domination by vasoconstriction[30]. Oxidative stress in the endothelium increases its permeability and promotes adhesion events[30]. Endothelial dysfunction can be quantified using flow mediated dilation (FMD), which measures the change in the diameter of an artery in response to blood flow.

Studies in mouse models have shown that reduced GPx1 activity results in endothelial dysfunction, increased inflammation and structural vascular abnormalities[17,22,23,29]. Insufficient GPx3 activity has been observed to increase platelet aggregation and NO responsiveness[5]. Functional variants in the GPx4 gene increase adhesion events in the endothelium, and these

polymorphisms have differential effects in Se deficient and Se replete environments[31]. Trx and TrxR play a role in regulating the metabolism of nitric oxide (NO), which is crucial to endothelial vasodilation[17]. There are no studies to our knowledge that have directly investigated the cross sectional association of selenium status and markers of endothelial function.

1.2.3 Arterial Stiffness

Arterial stiffness is characterized by the hardening of arteries. This occurs when there is an imbalance between collagen and elastin proteins in the endothelium[35]. This occurs naturally with aging and atherosclerosis, but is also influenced by genetic and lifestyle factors. Arterial stiffness is a primary risk factor for increased systolic blood pressure (SBP). It can be quantified using pulse wave velocity (PWV). Arterial stiffness leads to higher PWV.

There are very few existing studies that have investigated the relationship between selenium status and arterial stiffness. In a cross sectional study of patients at high risk for CVD, those within the bottom 10% of selenium intake had significantly higher PWV compared to the rest of the cohort[35]. There have been multiple RCTs that have shown reductions in arterial stiffness due to antioxidant supplementation, but few focus on selenium. An RCT from 2010 showed that an antioxidant supplement containing Se, Vitamin A, Vitamin E and Coenzyme Q10 improved arterial elasticity compared the placebo in a population of individuals at risk for CVD[53]. However, it is impossible to decipher the individual effects of each component in the supplement.

1.2.4 Detriment of High Selenium Status

Few studies have examined the high levels of Se that may increase the risk for CVD. The strongest evidence for detrimental effects of high Se status focus on insulin signaling. SEPP1 and GPx1 are both involved with insulin pathways[2,28]. Insulin induces ROS production when it binds to adipocytes in order to activate the insulin signaling cascade[2,28]. Both GPx1 and SEPP1 inhibit the production of ROS and therefore interfere with the insulin signaling cascade, which can lead to insulin resistance[2,28]. In a study of pregnant women, there was a significant positive association between GPx1 activity and fasting plasma glucose, insulin, and homeostatic model assessment of insulin resistance[36]. SEPP1 has been associated with hemoglobin A1C and fasting plasma glucose, which are measurements of glucose tolerance[37]. Another study showed that Korean patients with Type 2 Diabetes had significantly increased SEPP1 mRNA levels compared to those with normal insulin sensitivity[38].

In the National Prevention of Cancer trial, participants were randomly assigned 200µg of selenium per day or placebo. There was a significant increase in the incidence of Type 2 Diabetes Mellitus in participants who were within the highest tertile (>121.6 µg/L) of selenium status at baseline[39]. It is well established that insulin resistance and Type 2 Diabetes are associated with an increased risk for CVD[40].

2.0 METHODS

2.1 SAMPLE

The sample used in this study is from the Women and Infant Study of Health Hearts (WISH) cohort which was comprised of 702 White and African American women who gave birth between 1997 and 2002 in Pittsburgh, PA[41]. Women in WISH were recruited 4 to 12 years after giving birth to examine differences in subclinical atherosclerosis between women who had preterm births and those who had term births. Markers of cardiovascular disease were measured using cIMT, FMD and PWV[41]. Fasting blood samples were drawn for measurement of lipids and inflammatory markers. Investigators found that women with prior preterm births had significantly higher mean systolic and diastolic blood pressure, LDLc, triglycerides, apolipoprotein B and lower HDLc. Women with prior preterm births also had significantly higher CIMT than women with term births. Having at least one preterm birth is associated with an increased risk of CVD. These results suggest that the mechanism behind this could be related to chronic inflammation, and an earlier onset of atherosclerosis.

A random sample of 49 cases of mothers with preterm births and 51 controls (mothers with term births) were chosen for the analysis. A priori effect size calculations are shown in Appendix B. With 100 samples, there was at least 80% power to detect an absolute value of a correlation coefficient of 0.28 or greater using a two-sided test with α=0.05.

2.2 CVD BIOMARKER ASSESSMENTS

Protocols for these assessments in WISH have been previously reported[41]. Briefly, cIMT was measured at the baseline visit using B-mode ultrasound images of both the right and left distal common carotid artery, carotid bulb and internal carotid artery during diastole. Specialized software was then used to detect the edges of the lumen-intima and media-adventia and obtain intima media thickness values at each site. The mean values at each site were then averaged to obtain the mean IMT for each participant. cIMT is positively associated with the risk of CVD.

FMD was measured after 10 minutes of supine rest using B-mode ultrasound images of the right brachial artery. Ultrasound images were taken at rest and after 4 minutes of blood flow occlusion. The diameter of the artery at both time points was determined using specialized software. FMD was calculated by taking the percentage change in arterial diameters between the two time points. FMD is inversely associated with CVD risk.

PWV was measured by recording pressure waveforms in the right common carotid artery and right femoral artery simultaneously and calculating the distance between sites divided by the time the waveforms took to move between sites. This was performed 10 times and the mean PWV was reported. PWV is positively associated with the risk of CVD.

2.3 SELENIUM ANALYSIS

Serum samples were frozen at -80°C from the original study date until used for this analysis. The random sample of 49 cases and 51 controls from the WISH population was assayed for selenium detection using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a

Perkin-Elmer NeXION 300x ICP-MS (PerkinElmer Inc, Shelton, CT). This method is commonly used for analysis of serum and plasma selenium. The assays were completed at the University of Pittsburgh's Department of Geology and Planetary Science. Serum samples were thawed and 100µL of sample was diluted with 1.9mL of nitric acid and 16 µL of an internal standard was added. Samples were nebulized and introduced into the ICP plasma. ICP-MS uses argon discharge at extreme temperatures averaging 5000-10000K to ionize the samples. Results are presented in the accepted notation of µg/L. This assay was performed masked as to whether samples came from cases or controls.

2.4 STATISTICAL ANALYSIS

Descriptive statistics were calculated for baseline characteristics of the sample. Mean values and standard deviations are reported for normally distributed continuous values. Median values and the 25^{th} and 75^{th} percentile are reported for non-normally distributed continuous values. Frequencies and percentages are reported for categorical values. The distribution of selenium levels and markers of CVD were examined using the arithmetic mean, geometric mean, skewness, kurtosis and histograms. Distributions of continuous measurements were examined for normality and natural log transformed when skewed left. Frequency distributions with normal density functions were drawn for selenium levels and the natural log transformation of selenium levels (Figures 1 and 2). For variables with missing values, t-tests, Wilcoxon rank sum or chi square tests were used to determine whether differences of all other variables between those with the missing value and those without were statistically significant. Linear and quadratic relationships were examined between Se levels and cIMT, PWV and FMD using linear regression. Goodness

of fit was evaluated for both linear and quadratic relationships using R^2 values and residual plots. To test the hypothesis that CVD biomarkers are associated with selenium within the range of 55 to 145 µg/L, the same analyses were performed between selenium status and cIMT, PWV and FMD within and outside of this range. All statistical analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3.0 RESULTS

Table 1. Demographic and clinical variables of sample at time of blood draw (N=100)

Data Expressed as Mean(SD), Median (25^{th}, 75^{th} percentile) or Number (%)		*N Missing*
Race		2
African American	28 (28.6)	
White	70 (71.4)	
Age (Years)	29.7(6.6)	2
BMI (kg/m^2)	28.9(7.5)	2
Current Use of Blood Pressure Medication	6(6.1)	2
Current Use of Statins	2(2.0)	2
Diabetes	2(2.0)	2
Current Smoker	30(30.6)	2
Menopause	9(9.2)	2
Preterm Birth of Child	49(49.0)	0
Family History of Stroke	12(12.2)	2
Vitamin/Supplement Use	46(46.9)	2
Waist Circumference (cm)	95.6(16.7)	2
Total Cholesterol (mg/dL)	191(39.8)	2
LDL Cholesterol (mg/dL)	114(34.3)	2
Triglycerides (mg/dL)	95.0(67.0,136.0)	2
Diastolic Blood Pressure (mm Hg)	70.4(9.4)	2
Systolic Blood Pressure (mm Hg)	109(12.6)	2
Apolipoprotein B (mg/dL)	89.2(26.8)	2
CRP (mg/L)	2.1(1.0, 6.3)	2
Hemoglobin A1C (%)	5.1(0.5)	2
IL-6 (pg/mL)	1.7(1.0, 2.7)	21
Adiponectin (mg/L)	13.3(5.5)	2
Flow Mediated Dilation (%)	7.1(4.8,10.9)	45
Brachial Artery Diameter(mm)	3.4(0.8)	45
Pulse Wave Velocity (m/s)	7.6(1.4)	16
Carotid IMT (mm)	0.6(0.08)	12
Total MET Hours per Week	8.6(3.5, 16.0)	2

3.1 DESCRIPTIVE STATISTICS

Descriptive statistics for the sample at the time of blood draw are given in Table 1. The mean age for the sample was 29.7 years. The mean carotid IMT was 0.6 mm. The median FMD was 7.1%. The mean PWV was 7.6 m/s. LDL-c, CRP, IL-6, FMD and total MET hours per week had skewed distributions and were natural log transformed for analyses. 45 subjects had missing values for FMD, which was due to difficulty obtaining quality ultrasound images. Those that have measurements for FMD were compared to those without measurements for FMD. Student's t-test was used for normally distributed means, χ^2 tests for categorical variables and the Wilcoxon rank-sum test was used to compare smoking pack years, total MET hours per week, IL-6, CRP, apolipoprotein B and waist circumference. The only significant difference between the groups was the mean adiponectin values, which were 14.8 mg/dL and 11.5 mg/dL, respectively. Since the two groups appeared to be similar, the relationship between FMD and selenium levels was analyzed using 55 subjects.

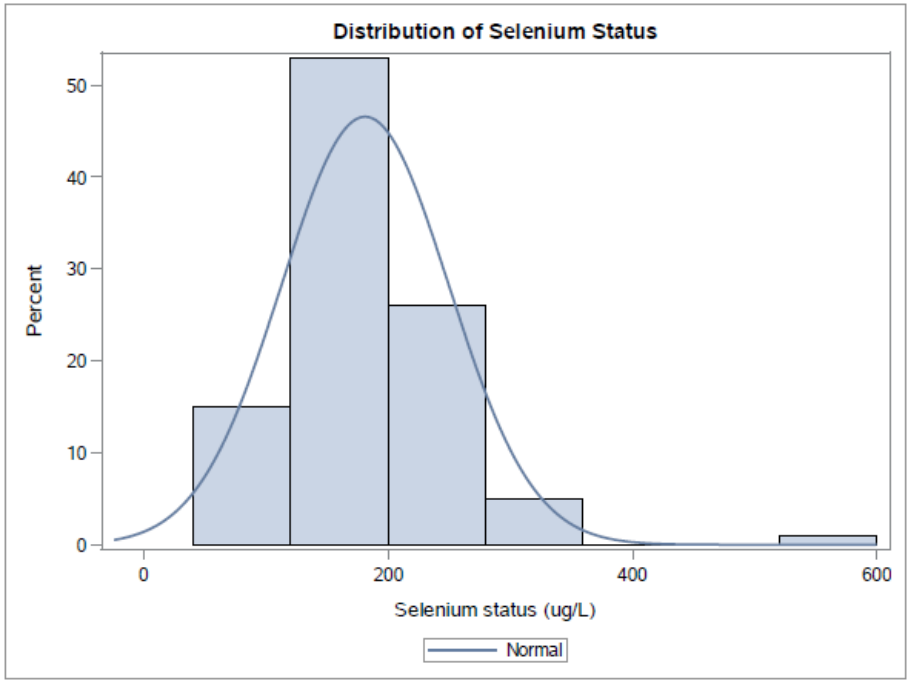

Figure 1. Distribution of Selenium status with normal density function

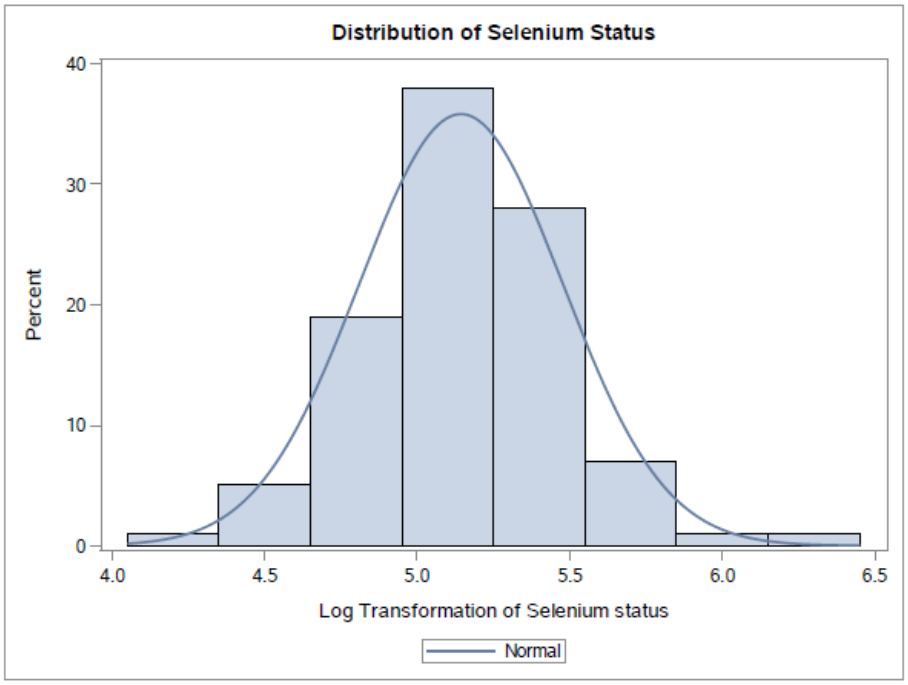

Figure 2. Distribution of ln(Selenium) levels with normal density function

Table 2. Distribution of Selenium levels

Percentile	***Selenium concentration (µg/L)***
Minimum	57.7
25th Percentile	142.8
Median	171.7
75th Percentile	210.0
Maximum	597.7
Range	
<55µg/L	0 (0%)
55-145 µg/L	26 (26%)
>145 µg/L	74 (74%)

3.2 SELENIUM DISTRIBUTION

The selenium distribution in this sample was skewed left, therefore selenium concentrations were ln transformed for these analyses. Figures 1 and 2 show the distributions of selenium levels and the ln transformed selenium levels respectively, with overlaid normal density functions. The median selenium level in this sample was 171.7µg/L (Table 2). This is a sufficient concentration for selenoprotein function, however this concentration is greater than the level shown to reduce cardiovascular disease risk[18,19]. The minimum selenium level in this sample was 57.7µg/L, which is within the range of values associated with low CVD risk[18,19]. The meta-analysis from 2015 found that the beneficial range for selenium status for cardiovascular health fell between 55-145µg/L, and only 26% of this sample fell within this range[19] (Table 2). The maximum selenium status was 597.7µg/L, this is an outlier, but is included in the analysis because it was not known to be inaccurate. This extreme value could possibly be due to supplementation,

but could be due to measurement error. Analyses excluding this outlier had similar results to the analyses in which it was included, so only the latter are reported.

Table 3. Regression model examining quadratic trends between Selenium levels and cIMT, PWV and FMD

	N	β_l[1]	P_l	β_q[2]	P_q
cIMT	88	0.58	0.14	-0.05	0.14
PWV	84	3.95	0.57	-0.44	0.51
FMD	55	2.17	0.53	-0.22	0.51

[1]β_l -regression coefficient for linear term, P_l- p value for linear term
[2]β_q -regression coefficient for quadratic term, P_q- p value for quadratic term

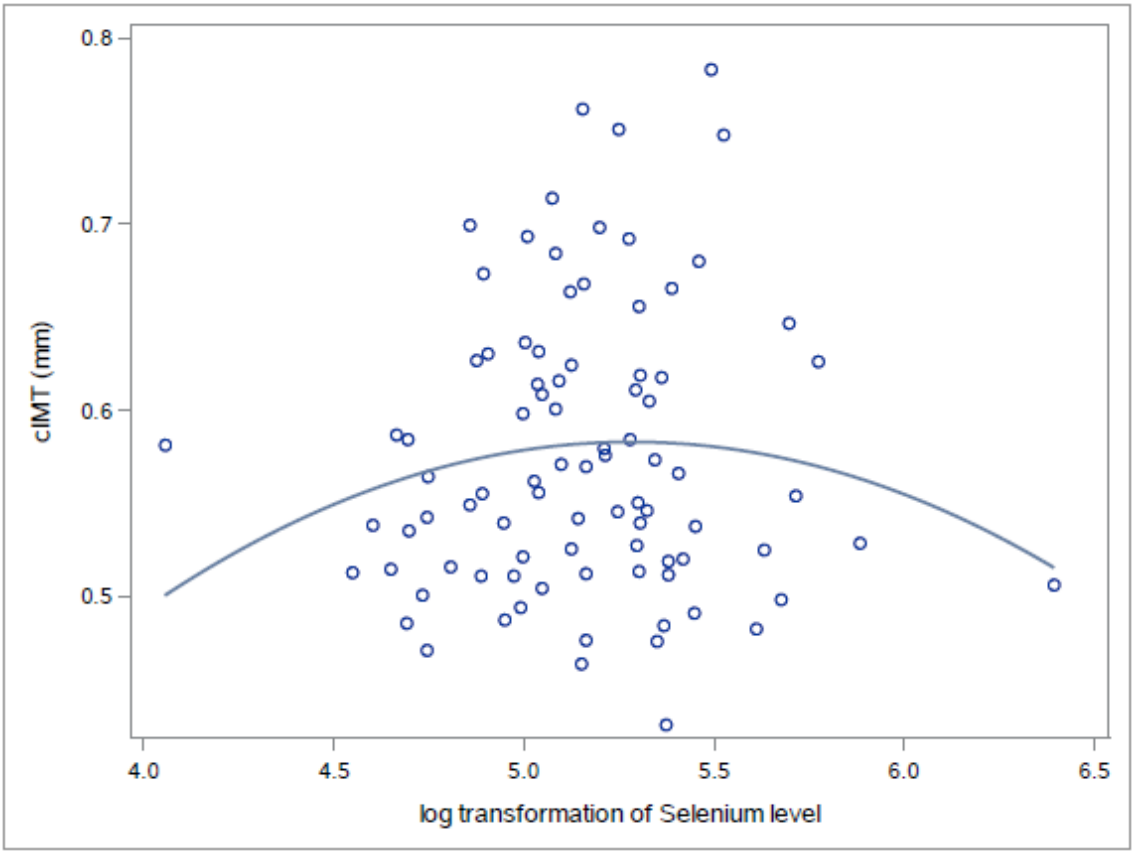

Figure 3. Scatter plot of Selenium level and cIMT

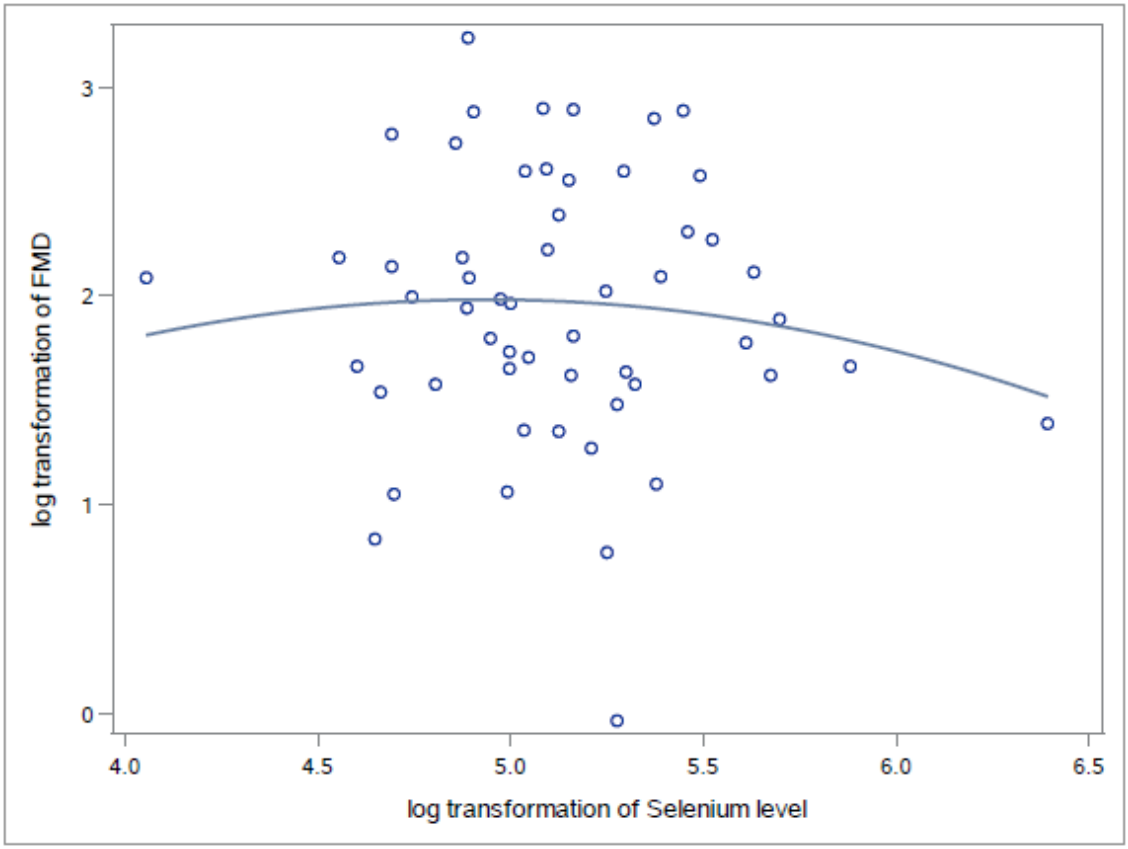

Figure 4. Scatter plot of Selenium level and FMD

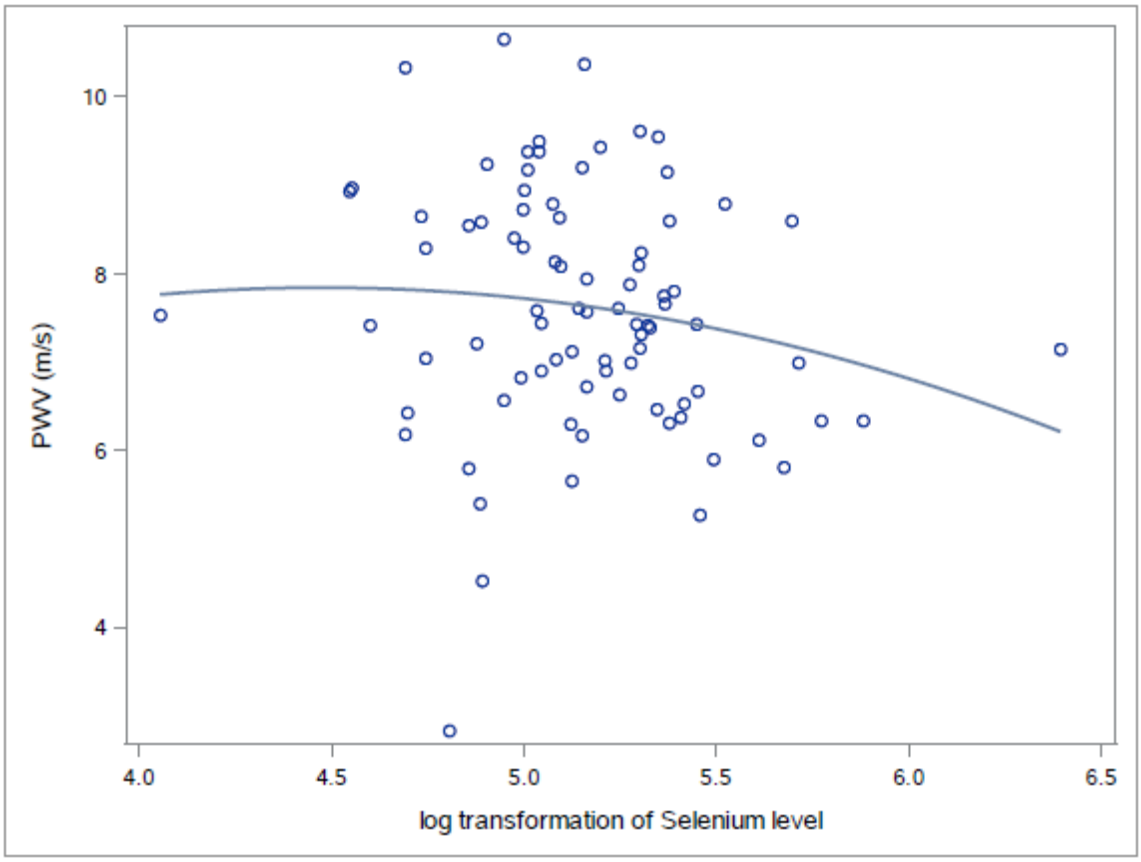

Figure 5. Scatter plot of Selenium level and PWV

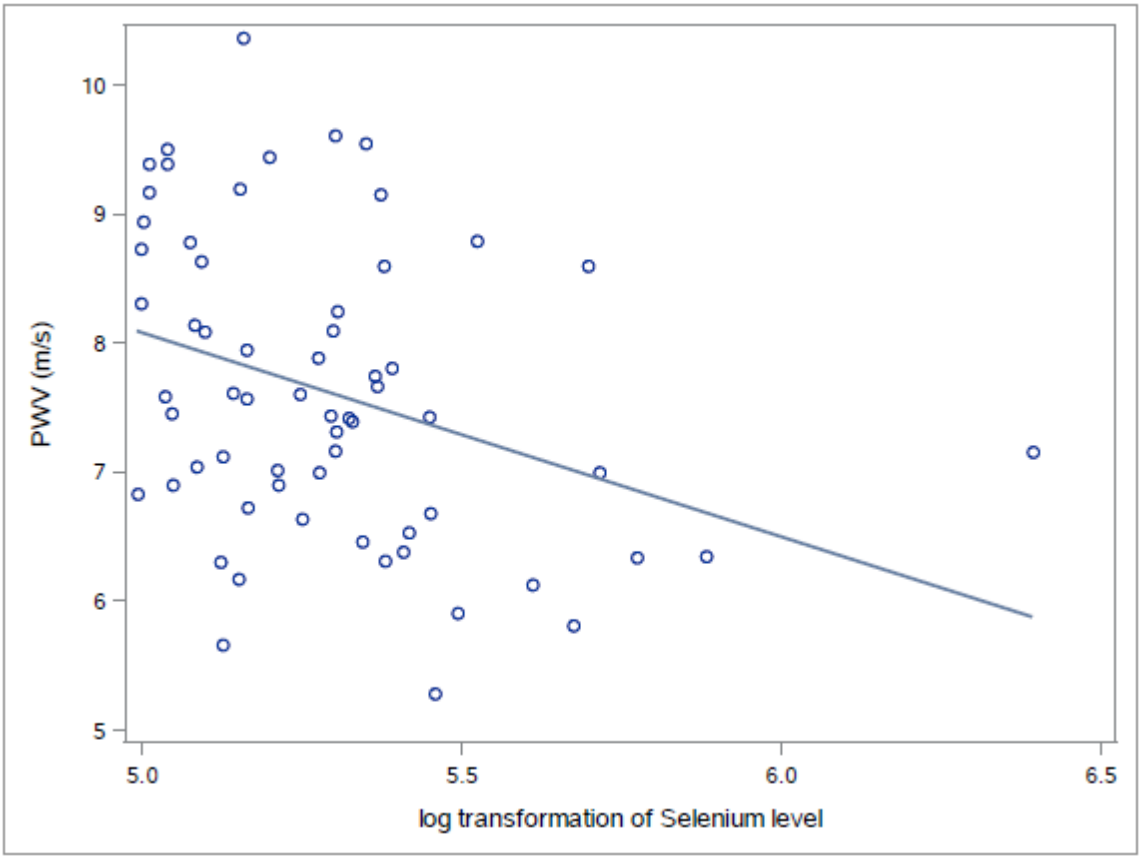

Figure 6. Scatter plot of Selenium levels >145 µg/L and PWV

3.3 ASSOCIATION BETWEEN SELENIUM LEVEL AND CIMT

Only 88 out of the 100 participants had measures for cIMT, which is positively correlated with CVD risk[5]. The scatter plot showing ln transformed selenium levels versus cIMT is shown in Figure 3. The quadratic regression curve is overlaid on the scatter plot. Examination of linear and quadratic trends using linear regression shows that the quadratic relationship between ln(Se) and cIMT has better goodness of fit than the linear relationship, though the regression coefficient for the quadratic model is not significant (p=0.14). This indicates that there is not a significant association between selenium concentrations and cIMT in this sample.

3.4 ASSOCIATION BETWEEN SELENIUM LEVEL AND PWV

Only 84 out of the 100 participants had measures for PWV, which is positively correlated with cardiovascular mortality[35]. The scatter plot showing ln transformed selenium levels versus PWV is shown in Figure 4. The quadratic regression curve is overlaid on the scatter plot. Examination of linear and quadratic trends using linear regression showed that the quadratic relationship between ln(Se) and PWV has better goodness of fit, though the regression coefficient for the quadratic model is not significant (p=0.51). This indicates that there is not a significant association between selenium status and PWV in this sample.

3.5 ASSOCIATION BETWEEN SELENIUM LEVEL AND FMD

Only 55 of the 100 participants had measures for FMD, which is inversely associated with CVD risk[30]. The scatter plot showing ln(Se) versus ln(FMD) is shown in Figure 5. The quadratic regression curve is overlaid on the scatter plot. Examination of linear and quadratic trends using linear regression showed that the quadratic relationship between ln(Se) and ln(FMD) has better goodness of fit, though the regression coefficient for the quadratic model is not significant ($p=0.51$). This indicates that there is not a significant association between selenium levels and FMD in this sample.

3.6 ANALYSES WITHIN AND OUTSIDE OF IDEAL SELENIUM RANGE

Only 26 of the 100 participants had selenium levels within the range of 55 to 145 µg/L (Table 2). There are no significant associations between Se levels and the CVD measures within the Se range of 55-145 µg/L. The other 74 participants had selenium concentrations greater than 145 µg/L. There are not significant associations between selenium levels >145 µg/L and cIMT (n=65) or FMD (n=37). The scatter plot showing ln transformed selenium status versus PWV in those with selenium >145 µg/L is shown in Figure 6. The linear regression function is overlaid over the scatter plot. Interestingly, for selenium levels greater than 145 µg/L, there is a significant negative linear relationship between ln(Se) and PWV ($\beta=-1.58$, $p=0.006$).

4.0 DISCUSSION

There are no significant associations between selenium status and either cIMT, PWV or FMD in this sample of 100 women. Only 26% of the sample fell within the range of Se levels that is associated with a decreased risk of cardiovascular disease, the remaining majority of the sample had Se levels above 145 µg/L[19]. There is no established cut point that defines Se deficiency, but studies of Keshan disease in China showed that the disease did not occur in communities where Se concentrations averaged 21 µg/L[43]. Therefore, it can be assumed that no individuals were deficient in selenium, since the minimum Se status in the sample used for the current analysis was 57.7 µg/L. This supports previous research that North Americans, on average, have sufficient intakes of Se[2,3,6,8].

The results of these analyses do not support that Se is associated with CVD risk in this sample. However, only 26 participants have Se levels in the range expected to reduce CVD risk. The CVD biomarkers indicate that there is low CVD risk, which limits the ability to detect a relationship between Se levels and cIMT, PWV and FMD. In the sub-analyses examining the correlations within and outside of this range, there is a significant association only between Se levels above 145 µg/L and PWV. This indicates that higher selenium levels are associated with lower PWV, which is associated with a lower risk for CVD. This conflicts with the findings in previously published research and bears further examination[18,19].

The lack of a statistically significant association between Se and the CVD markers in this sample may be due to a lack of power. Effect size calculations were computed based on the sample size of 100 individuals for which selenium levels could be measured. However, only 88 participants had measurements for cIMT, 84 for PWV, and 55 for FMD. The observed correlations

between Se status and cIMT and FMD are close to zero. The relationship between PWV and Se level is stronger, particularly within the subgroup of participants who have Se concentrations greater than 145 µg/L.

This sample of women had a mean age of 29.7 years, which is a young group to study cardiovascular disease since age is positively associated with the risk of CVD[44-46]. The sample also appears to have good cardiovascular health, with low blood pressure, total cholesterol and a low prevalence of diabetes. A meta-analysis in 2013 described ideal cardiovascular health metrics for young adults with a mean age of 29.5 years. Their ideal systolic blood pressure is 120 mmHg, ideal diastolic blood pressure is 80 mmHg and ideal total cholesterol is <200mg/dL[44].

Because the sample used in this analysis was young and relatively healthy, measures of the three CVD markers are associated with minimal CVD risk. The median FMD was 7.1%. In a study of young, healthy Finnish adults with a mean age of 31.6 years, the mean FMD was 7.8%[45]. The mean PWV in this sample was 7.6m/s. In a meta-analysis of healthy adult females with a mean age of 37, the mean PWV among was 7.7m/s[47]. The mean cIMT was 0.6mm in this sample. A cohort of healthy young American adults with a mean age of 29.5 years was previously reported to have a mean cIMT of 0.67mm[46]. PWV and IMT are positively associated with age, while FMD is inversely associated[46.47,55]. Based on the previous literature of populations with similar age ranges, this sample, on average, had minimal CVD risk.

The only significant association between selenium status and CVD is between selenium levels >145 µg/L and PWV. Selenium concentrations may only be a predictor of CVD risk at older ages. Additionally, the Zhang meta-analysis did not have sufficient evidence to examine the association of CVD risk and selenium status above 165 µg/L, but the relationship was null between 145 and 165 µg/L[19]. They hypothesized that the U-shaped association between Se levels and CVD

risk is true[19]. These analyses show a possible beneficial relationship between higher Se status and arterial stiffness, which would not support the U-shaped association.

This small pilot study has limitations. The small sample size limits power to observe associations between selenium levels and the measures of CVD. The sample has limited diversity, being only White and African American females. The young age of the sample limits the ability to detect atherosclerosis, arterial stiffness and endothelial dysfunction. Factors which may confound any associations were not adjusted for in the analysis.

However, strengths of this study include the use of an objective measure of selenium status. CVD risk is examined in a young, fairly healthy sample, which can help to determine mechanisms for prevention. This is the first study to examine the cross-sectional relationship between selenium status and cIMT, PWV and FMD in a healthy population.

Cardiovascular disease is one of the top causes of morbidity and mortality worldwide[44]. It has been established that selenium levels, within a certain range, are associated with a lower risk of CVD[18,19]. The field of CVD prevention and treatment would benefit from a greater understanding of the biological pathways that lead to arterial stiffness, endothelial function and atherosclerosis. This study shows that higher Se concentrations may be associated with PWV, a marker of arterial stiffness. Increased arterial stiffness is one of the primary risk factors for increased systolic blood pressure. Because arterial stiffness is reversible, there may be a potential for therapeutic supplementation of selenium.

Overall, this sample of women had adequate levels of selenium status. When restricted to selenium concentrations greater than 145 µg/L, there is a significant inverse relationship between Se and PWV. This association has not been examined in a healthy population previously so it may be useful to examine the relationship in larger studies with a more diverse population. This

suggests many future directions. The association between Se and PWV should be examined in older people and among individuals with higher risk for CVD. This association should also be studied in male populations, as males have increased risk for CVD at younger ages compared to women. It is also important to study these relationships in populations that have Se levels less than 55 µg/L. There may be stronger associations with CVD risk when selenium concentrations are inadequate. The quadratic trend between Se levels and CVD risk needs to be validated. There are currently few studies that examine the cardiovascular risks and benefits of selenium supplementation. The U-shaped association may not appear for individual markers of CVD, as is shown in this analysis with PWV. There may also be other markers of CVD that may have stronger associations with selenium concentrations. Additionally, there are known variants in the GPx1, GPx3 and GPx4 genes that affect CVD risk, so genotyping of these variants should also be encouraged in studies of cardiovascular health[22,24,25,31].

APPENDIX A: PREVIOUS STUDIES OF SELENIUM STATUS AND CVD RISK

Tables 4 and 5 contains a review of previous observational studies, RCTs and meta-analyses of studies investigating selenium status or supplementation in association with various CVD risk factors. Table 4 contains studies with significant results and Table 5 contains studies with null results.

Table 4. Selected Selenium studies with significant results

Study	Baseline Range of Mean of Se Status	Population	Type of study	Method of Se measurement	Results
NHANES (2003-2004) (Laclaustra et al)	136.7ug/L	Free living adults >40 in USA	Cross-sectional analysis	Serum	Positive correlation of Se status w/ total cholesterol, LDL-c. Positive correlation of Se status w/ HDL-c to plateau at 120ug/L. U shaped association of Se and triglycerides.
AtheroGene (Lubos et al)	70ug/L	Patients w/ ACS or SAP in Germany	Cohort w/ 6 year follow-up	Serum	Highest tertile of Se status associated w/ decreased risk of death compared to lowest tertile.
ULSAM (Helmersson et al)	77ug/L	Population based cohort of Swedish men >50 years old	Cohort w/ 27 year follow-up	Serum	Inverse association of Se and isoprostanes and prostaglandins
Zhang et al (2015)	N/A	Various studies from USA and Europe	Meta-analysis of 16 prospective studies and 16 RCTs	Blood	Significant decrease in CVD risk within Se range of 55-145ug/L in observational studies. No effect of Se supplementation in RCTs.
Flores-Mateo et al (2006)	N/A	Various studies from USA, Europe and China	Meta-analysis of 25 observational studies and 6 RCTs	Blood and toenail	Se status inversely associated with CHD risk in observational studies. No effect of Se supplementation in RCTs.
Shargorodsky et al	Not indicated	Patients w/ >2 CVD risk factors in Israel	RCT: Supplementation of mixture containing 100ug Se/day or placebo for 6 months	Not indicated	Antioxidant group had significantly improved arterial elasticity, HbA1c and HDL-c.
Alehagen et al	Not indicated	Healthy elderly individuals in Sweden	RCT: Supplementation of mixture containing 200ug Se/day or placebo for 48 months	Not indicated	Supplementation associated with significantly reduced mortality

*ACS= Acute coronary syndrome, SAP=stable angina pectoris, CHD=coronary heart disease

Table 5. Selected Selenium studies with null results

Study	Baseline Range of Mean of Se Status	Population	Type of study	Method of Se measurement	Results
Myung et al	N/A	Various	Meta-analysis of 7 Se supplementation RCTs	N/A	No effect of Se supplementation of CVD risk
Hawkes et al	146ug/L	Healthy men 18-45 years old in USA	RCT: Supplementation of 300ug Se/day or placebo for 48 weeks	Plasma and erythrocytes	No effect of Se supplementation on FMD
CARDIA (Xun P et al)	0.48-1.98 ug/g	Healthy 20-32 year olds in USA	Cohort w/ 18 year follow-up	Toenail	No association between Se and cIMT or CAC

APPENDIX B: POWER AND EFFECT SIZE CALCULATIONS

Effect size calculations were performed a priori with the assumptions of 100 samples, 2 sided α=0.05 for Pearsons's correlation tests against the hypothesis ρ_1=0.

Table 6. Effect size calculations for Pearsons's correlation coefficients

Power	**$\lvert\rho_1\rvert$**
0.99	0.4
0.87	0.3
0.52	0.2
0.17	0.1

B.1 POST HOC POWER ANALYSIS

Measures for the CVD markers were missing for some subjects, so power calculations were performed for Pearson's correlation coefficient with the actual sample sizes for each measurement and two sided α=0.05.

Table 7. Post-hoc Effect Size Calculations for Pearson Correlation Coefficients

	N	$\|\rho_1\|$	***Power***
IMT	88	0.34	0.9
IMT	88	0.29	0.8
IMT	88	0.26	0.7
PWV	84	0.34	0.9
PWV	84	0.30	0.8
PWV	84	0.27	0.7
FMD	55	0.42	0.9
FMD	55	0.37	0.8
FMD	55	0.33	0.7

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