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# The Efficacy of Curcumin Supplementation to Improve Age-Related Arterial Dysfunction in Healthy Middle-Aged and Older Adults

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# THE EFFICACY OF CURCUMIN SUPPLEMENTATION TO IMPROVE AGE-RELATED ARTERIAL DYSFUNCTION IN HEALTHY MIDDLE-AGED AND OLDER ADULTS

by

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The efficacy of curcumin supplementation to improve age-related arterial dysfunction in healthy middle-aged and older adults

Thesis directed by Professor Douglas R. Seals

Cardiovascular diseases (CVD) are the primary cause of death in developed societies. Advancing age is the major risk factor for CVD, with a ~70% prevalence of CVD in adults over 60 years of age. This increase in CVD risk with aging is due primarily to adverse changes to arteries, in particular, the development of two key physiological changes: vascular endothelial dysfunction, characterized by a decline in nitric oxide-mediated endothelium-dependent dilation, and increased stiffness of large elastic arteries. Age-associated arterial dysfunction is driven primarily by the presence of oxidative stress and inflammation.

With the number of adults over the age of 60 expected to double by 2050, preventative and intervention strategies are needed. Curcumin, the active ingredient in the Indian spice turmeric, is a naturally occurring phenol with antioxidant and anti-inflammatory properties. Additionally, 4 weeks of curcumin supplementation improved macrovascular endothelial function and large elastic artery stiffness in older mice.

The goal of this dissertation was to test the hypothesis that curcumin supplementation would improve micro- and macrovascular endothelial function in healthy middle-aged and older adults and these improvements would be associated with increased nitric oxide bioavailability and reduced oxidative stress and inflammation. A secondary hypothesis was curcumin supplementation would improve regional and local large elastic artery stiffness in healthy middle-aged and older adults.

Thirty-nine healthy men and postmenopausal women 45 to 79 years of age were randomized to curcumin (2000 mg/day Longvida) or placebo supplementation for 12 weeks. Curcumin supplementation improved microvascular endothelial function by increasing nitric oxide and reducing vascular oxidative stress, and improved macrovascular endothelial function. In contrast, curcumin had no effect on circulating markers of oxidative stress and inflammation. Secondly, large elastic artery stiffness was assessed in the same individuals before and after 12 weeks of curcumin or placebo supplementation. Curcumin had no influence on regional or local large elastic artery stiffness.

Taken together, these results indicate that curcumin supplementation improves vascular endothelial function, a key antecedent to CVD, and therefore may be a promising prevention for the development of CVD in healthy middle-aged and older adults.

## Dedication

This is dedicated to the one I love, my husband Keli. Words aren't enough to express how grateful I am to have you as my rock, who has supported me, encouraged me, and above all *always* believed in me. I couldn't have done it without you and am beyond excited for our futures working side by side as physician scientists.

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## List of publications

### Research Articles

**Santos-Parker JR**, Santos-Parker KS, Martens CR, McQueen MB, Seals DR. Circulating proteome: effects of age, sex, and exercise and relation to physiological function. In preparation.

*Johnson LC, ***Santos-Parker JR**, Martens CR, Strahler TR, Bassett CJ, McQueen MB, Seals DR. Curcumin supplementation alters the plasma metabolome and improves vascular endothelial function in middle aged and older adults. In preparation.
***Co-first authors**

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# Chapter II

## Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality in developed societies with advancing age as the major risk factor for CVD[1-3]. Increased risk of CVD with age is thought to result from adverse changes to arteries, making them more susceptible to diseases. Age-associated arterial dysfunction is primarily due to the development of vascular endothelial dysfunction, as measured by impaired endothelium-dependent dilation, and stiffening of the large elastic arteries[4].

Micro- (resistance artery) and macrovascular (conduit artery) endothelial function decline with advancing age[5-11] and each is independently predictive of future risk of cardiovascular events and mortality[12-15]. A key mechanism mediating the development of age-related endothelial dysfunction is reduced bioavailability of the vascular protective and vasodilatory molecule, nitric oxide[10, 16-18]. Decreased nitric oxide bioavailability with age is driven, in part, by the presence of oxidative stress, defined as an increase in reactive oxygen species relative to antioxidant defenses, and chronic low-grade inflammation[8, 19-21].

Large elastic arteries stiffen with advancing age[22-25], a characteristic associated with a higher risk of cardiovascular-related mortality[26-28]. Changes in vascular smooth muscle tone and structural components of the arterial wall are thought to be the predominate factors contributing to the increase in stiffness[4, 29, 30], driven by reductions in nitric oxide bioavailability and increases in oxidative stress and inflammation[31-33].

Thus, novel strategies to reduce vascular endothelial dysfunction and large elastic artery stiffness by increasing nitric oxide bioavailability and reducing arterial oxidative stress and inflammation in aging adults have the potential to prevent the development of CVD, and therefore, are of high biomedical priority.

One promising intervention is oral curcumin supplementation. Curcumin is a safe and naturally occurring phenol that is the active ingredient in the Indian spice turmeric, giving it its yellowish color. Curcumin has been reported to have antioxidant and anti-inflammatory properties *in vitro* and *in vivo*[34-42]. Research assessing the effects of curcumin on primary aging has been limited. 4 weeks of curcumin supplementation was reported to ameliorate age-associated vascular endothelial dysfunction and large elastic artery stiffness in older mice by increasing nitric oxide bioavailability and reducing oxidative stress and inflammation[43]. Additionally, 8 weeks of curcumin supplementation improved macrovascular endothelial function and carotid artery compliance, a measure of large elastic artery stiffness, in healthy middle-aged and older Japanese postmenopausal women[44, 45].

As such, curcumin holds promise as a safe and effective intervention for the development of age-related arterial dysfunction and the prevention of age-associated CVD. However, more research is needed to examine the efficacy of curcumin supplementation on micro- and macrovascular endothelial function and large elastic artery stiffness in both men and women and the mechanisms therein involved.

The goal of this dissertation was to test the hypothesis that curcumin supplementation would improve micro- and macrovascular endothelial function in healthy middle-aged and older adults and these improvements would be associated

with increased nitric oxide bioavailability and reduced oxidative stress and inflammation. A secondary hypothesis tested if 12 weeks of curcumin supplementation would improve regional and local large elastic artery stiffness in healthy middle-aged and older adults.

## Specific aims

**Specific Aim 1 (Chapter IV):** To determine if 12 weeks of curcumin supplementation improves micro- and macrovascular endothelial function in healthy middle-aged and older adults

**Specific Aim 2 (Chapter IV):** To determine if improvements in vascular endothelial function with curcumin supplementation are mediated by increased nitric oxide bioavailability

**Specific Aim 3 (Chapter IV):** To determine if improvements in vascular endothelial function with curcumin supplementation are associated with reduced oxidative stress and inflammation

**Specific Aim 4 (Chapter V):** To determine if 12 weeks of curcumin supplementation decreases local and regional large elastic artery stiffness in healthy middle-aged and older adults

# Chapter III

*Portions of this chapter were published previously in Advan in Physiol Edu 38: 296-307, 2014 (Santos-Parker JR, LaRocca TJ, and Seals DR)*

## Review of literature

### Significance

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in developed societies[1, 2]. Advancing age is the primary risk factor for CVD, with >90% of CVD occurring in middle-aged and older adults[1, 3]. With the aging of the "baby boomer" generation (adults born between 1946 and 1964), the number of older adults in the United States is expected to double between now and 2050[46]. As a result, a new epidemic of "boomer-driven" CVD is projected during this period. Indeed, a 2011 American Heart Association policy statement predicted that without effective intervention, largely as a result of population aging, 40% of United States adults will have one or more forms of CVD by 2030 and medical costs will triple[47]. To address this impending biomedical challenge, it is essential to identify and implement preventive strategies and interventions that will delay and reverse the development of age-associated CVD.

Aging leads to increased risk of CVD primarily through the development of arterial dysfunction, which is largely attributable to two physiological changes: the development of micro- and macrovascular endothelial dysfunction and stiffening of the large elastic arteries (the aorta and carotid arteries)[4].

## Vascular endothelial function and aging

The endothelium, a monolayer of cells lining the circulatory system, was once thought to be an inert physical barrier between blood and tissue[48]. It is now understood that the endothelium is a dynamic and heterogeneous organ that plays a critical role in maintaining vascular homeostasis and is equipped to respond quickly to changes in biological needs. The endothelium acts as a gatekeeper for the regulation of nutrient delivery and releases vasoactive factors to regulate blood cells, vascular tone, and blood flow[48]. The endothelium is continuously receiving, transducing, and delivering a myriad of signals through the presence of multiple receptors for proteins, lipids, hormones, metabolites, and constant cell-cell communication through junctions and receptors. In healthy normal individuals the endothelium is antithrombotic, antifibrinolytic, anti-inflammatory, and antiproliferative[16, 48]. Advancing age shifts the endothelium to a vasoconstrictor, prothrombotic, profibrinolytic, proinflammatory, and proliferative phenotype. *Endothelial dysfunction* refers to the functional alteration in the normal endothelial phenotype that contributes to the development of CVD[16, 49, 50].

***Role of nitric oxide in the vasculature.*** The endothelium synthesizes and releases a wide array of vasoactive factors in an autocrine and paracrine fashion to regulate vascular tone and maintain vascular homeostasis. The predominant endothelium derived vasodilator in the systemic arterial system is nitric oxide (NO). NO is a gaseous free radical product generated in the presence of oxygen by the oxidation of L-arginine to L-citrulline with the required cofactors nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin ($BH_4$), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), heme, and zinc[51]. NO acts as a potent endothelium-derived

vasodilator by binding soluble guanylyl cyclase in vascular smooth muscle cells, initiating a cascade of events that results in vascular smooth muscle relaxation. NO has a multitude of other cellular functions including anti-inflammatory, antithrombotic, and antiproliferative properties[48, 52]. Its high biological reactivity and short half-life necessitate tight control to ensure the correct amount synthesized and precise timing of release[51].

NO is generated from the NO synthase (NOS) family of enzymes. NOS are multi-domain enzymes with binding sites for heme, L-arginine, $BH_4$, NADPH, FMN, FAD, calmodulin, and Heat shock protein 90 (Hsp90)[53]. Specifically, the endothelial NOS (eNOS) isoform is the predominantly expressed NOS isoform in healthy endothelial cells[51] and is constitutively expressed and regulated by intracellular calcium levels and phosphorylation to produce low levels of NO. While eNOS is constitutively active, its expression and activation can be regulated transcriptionally (via epigenetic modifications), post-transcriptionally, and post-translationally. Post-translational modifications include lipidation, phosphorylation, S-nitrosylation, acetylation, and protein-protein interactions. Phosphorylation of Thr495 and S-nitrosylation of Cys94 and Cys99 reduces eNOS activity, phosphorylation of Ser1177 enhances activity, and acetylation and deacetylation of lysines can have opposing effects depending on the specific lysine modified[51, 54]. Furthermore, caveolin-1 interaction downregulates eNOS activity, whereas Hsp90 and calmodulin interaction upregulate activity[54].

**Assessment of vascular endothelial function**

Vascular endothelial function is commonly assessed by measuring endothelium-dependent dilation (EDD), the dilation of blood vessels in response to a stimulus

(mechanical or chemical) that evokes NO production via the activation of the enzyme eNOS. NO released by the endothelium diffuses into the surrounding smooth muscle, leading to relaxation and consequent dilation of the artery and increased blood flow that can be measured by several methods[16, 55-57]. The best characterized and most widely used techniques for assessing EDD in humans include 1) ultrasound assessment of brachial artery dilation in response to an increase in blood flow (mechanical stimulus) produced by temporary forearm ischemia (brachial artery flow-mediated dilation [FMD]) and 2) increases in forearm blood flow in response to acetylcholine infusion ($FBF_{ACh}$; pharmacological stimulus)[42]. Whereas brachial artery FMD is considered a measure of macrovascular (conduit) EDD, $FBF_{ACh}$ is considered an indicator of microvascular (resistance vessel) EDD[16]. In rodents, isolation of arteries (the aorta, carotid arteries, etc.) to measure the change in dilation in response to ACh incubation (pharmacological stimulus) is used to assess EDD[58-62].

Coronary EDD in response to ACh infusions (NO mediated) is reduced in large epicardial coronary arteries and coronary resistance vessels with age in healthy normal adults[63]. Some studies observe coronary vasoconstriction in response to ACh in older adults or individuals with coronary risk factors[64, 65]. Peripheral conduit artery EDD (brachial artery FMD) has been shown to correlate with coronary endothelial function (EDD assessed via intracoronary infusions of ACh), suggesting that impaired EDD is a systemic event and peripheral EDD can be used as a non-invasive and adequate surrogate for coronary EDD[66].

Both brachial artery FMD and $FBF_{ACh}$ are reduced with advancing age even in adults free of major CVD risk factors or clinical disease, indicating endothelial

dysfunction as a primary effect of aging[5-11]. In a cross-sectional study, brachial artery FMD was shown to progressively decline after age 40 in healthy men and after age 50 in healthy women, whereas in women the rate of decline was much steeper[5]. Brachial artery FMD has been reported to be 33-50% lower in older adults compared to young adults[7, 8, 67-69]. $FBF_{ACh}$ progressively declines (1.8% per year) with age in healthy normotensive men. However, in women a slight decrease (0.5% per year) in $FBF_{ACh}$ is observed until age 50, after which women >50 years old experience more rapid declines in EDD (2.1% per year) than men. After age 60, no sex-related differences in $FBF_{ACh}$ decline are observed[9]. $FBF_{ACh}$ has been reported to be 25-65% lower in healthy older adults compared to their young counterparts[6, 9-11, 13, 70, 71]. Within the same older healthy men who had age-related impaired $FBF_{ACh}$, no age-related difference in femoral blood flow in response to ACh was observed[13].

Importantly, both $FBF_{ACh}$ and brachial artery FMD are predictive of future risk of a cardiovascular event or mortality and remain significant predictors when adjusted for other coronary risk factors[12, 14, 15]. In addition, these measures of EDD add prediction capability when used in conjunction with Framingham risk score[12]. A meta-analysis suggests that a 1% decrease in brachial artery FMD is associated with a 13% increase in risk for future cardiovascular events[72]. However, brachial artery FMD and $FBF_{ACh}$ within individuals are not correlated, suggesting differences in vascular smooth muscle tone regulation between conduit and resistance vessels and that different measures of EDD may have relevance to different aspects of CVD[73].

Preclinical and clinical data support that age-related impaired EDD is mediated by declining NO bioavailability[16]. The inhibiting effect of the NOS inhibitor *NG*

monomethyl-L-arginine (L-NMMA) with ACh is progressively lower with advancing age in normotensive adults[10]. In addition, L-NMMA has less of an inhibitory effect in older adults compared to young adults[16, 17], demonstrating reduced NO bioavailability with age.

EDD measures are commonly compared to measures of endothelium-independent dilation to isolate endothelial function from vascular smooth muscle sensitivity to NO. The best characterized and most widely used techniques for assessing endothelium-independent dilation in humans include 1) the vasodilatory response to the NO donor nitroglycerin and 2) the increases in forearm blood flow in response to the NO donor sodium nitroprusside ($FBF_{SNP}$), infusion. Nitroglycerin response is considered a measure of macrovascular (conduit) endothelium-independent dilation and is compared to brachial artery FMD, whereas $FBF_{SNP}$ is viewed as an indicator of microvascular (resistance) endothelium-independent dilation and is compared to $FBF_{ACh}$. Most research supports no change in vasodilatory response to NO donors (nitroglycerin, SNP) in healthy adults with advancing age[5-10, 67, 68, 71, 74, 75]. While some research indicates a decline in endothelium-independent dilation response with age, the change is not as great as the reduction in age-related EDD[9-11]. These studies also included cohorts with major cardiovascular risk factors, potentially explaining the discrepancies to healthy aging populations.

**Large elastic artery stiffness and aging**

With each contraction of the left ventricle, a bolus of blood is ejected into the arterial system. The aorta acts as a pressure buffer, dampening the oscillatory pulse of blood ejected by the left ventricle by expanding and storing the bolus of blood during

systole and recoiling during diastole to propel blood to the peripheral circulation. In healthy young individuals, the aorta is compliant when receiving the blood ejected from the left ventricle, minimizing the workload of the heart. This ejection of blood from the left ventricle generates a forward moving pressure wave, with the amplitude and velocity of the wave dependent on the elasticity of the aorta. As the forward moving pressure wave travels through the arterial tree it encounters changes in impedance, resistance to pulsatile blood flow, due to branching, tapering, and changes in structural composition of the arterial wall. These transitions in impedance result in the generation of reflected waves that summate to form a single reflected wave that returns to the heart. In healthy young individuals, this reflected wave returns to the heart during diastole, elevating diastolic pressure[33, 76].

Coronary arteries that supply the heart are closed off during systole (ventricular contraction) and blood supply to the heart occurs during diastole (ventricular relaxation). Myocardial oxygen supply is determined by the duration of diastole, specifically the patency of coronary arteries, and the pressure gradient across the myocardial bed. In young adults, increased diastolic pressure increases coronary perfusion pressure, thereby increasing blood flow and oxygen delivery to the heart tissue. Furthermore, in healthy young individuals an impedance mismatch between the central and peripheral arterial systems allows for continuous minute reflection of waves as the pressure wave moves forward, reducing the amplitude of the pulsatile pressure into high-flow (low-impedance) sensitive organs, such as the brain, heart, and kidneys[76, 77].

However, with advancing age the large elastic arteries stiffen (i.e. become less compliant). This in turn results in the left ventricle working harder to eject the same

amount of blood into a stiffer aorta and ensuing in a higher amplitude of the forward pressure wave (an increase in systolic blood pressure). Furthermore, the forward pressure wave travels at a greater velocity along the stiffer large elastic artery resulting in the reflected wave returning sooner to the heart, now during systole. Earlier return of the reflected wave has two negative impacts—first, increasing systolic blood pressure and further increasing the workload of the heart (the left ventricle now must overcome a greater pressure to eject blood), and second, reducing diastolic blood pressure and myocardial perfusion. This creates a mismatch between the workload of the heart and its oxygen supply—the heart now has to work harder and requires more oxygen, but with less oxygen available[33]. These changes can lead to left ventricular hypertrophy and an increased risk of myocardial infarction and heart failure[4, 29, 78]. Also with age, the impedance mismatch between the central elastic arteries and periphery is diminished and minute continuous wave reflections no longer occur as regularly, resulting in a larger pulsatile forward pressure wave penetrating into the vulnerable high flow-dependent organs and causing microvascular tissue damage[4, 29, 77, 78].

The cardiovascular system is arranged into a network of blood vessels with the overall function to deliver oxygen, nutrients, and hormones to tissues and remove metabolic wastes. Blood vessels are composed of three layers. The innermost layer, the tunica intima, is composed of a monolayer of endothelial cells anchored to the basal lamina. The middle layer, the tunica media, is primarily composed of vascular smooth muscle cells, elastin and collagen fibers, and ground substance. The outermost tunic, the tunica adventitia, is composed of loose connective tissue including the extracellular matrix, fibroblasts, immune cells, and adrenergic nerves[51, 79]. The three distinct

anatomical regions of the arterial system include large elastic arteries, muscular arteries, and arterioles. Composition and structure of blood vessels in each anatomical region vary to support each region's respective function[80].

Age-associated stiffening of the large elastic arteries is mediated by both functional changes and structural changes in the arterial wall. Structural changes include extracellular matrix remodeling (deposition of the load-bearing protein collagen and decreases in the elasticity conferring protein elastin) and the formation of advanced glycation end products (AGEs), which crosslink these structural proteins and confer additional stiffening[4, 23, 29, 81]. Functional changes with age that contribute to arterial stiffening result from increased vascular smooth muscle tone due to increased sympathetic nervous and renin-angiotensin-aldosterone system activities and endothelium-derived vasoconstrictors, in addition to decreased endothelium-derived vasodilators[32, 82].

**Assessment of arterial stiffness**

Arterial stiffness is commonly assessed as the velocity of the arterial pressure pulse wave traveling through a defined arterial segment. The pressure waves' velocity (pulse wave velocity, PWV) is dependent on arterial material stiffness (the material's intrinsic ability to resist distension when a force is applied to it), wall thickness, diameter, and blood density[80, 83]. Specifically, PWV can be determined non-invasively via applanation tonometry with electrocardiogram gating at the R wave and is calculated as the distance (d) between two arterial sites divided by the arterial pressure wave transit time ($\Delta t$) between recorded arterial pressure waves at each site. The faster the pressure wave travels between arterial sites, the stiffer the artery[84].

PWV between the carotid and femoral arteries is used as an estimate of aortic stiffness (aortic PWV) due to the path the pulse wave travels through the aorta. Aortic PWV is the current gold standard for assessing arterial stiffness due to the role the aorta plays in cushioning pulsatile blood ejected from the left ventricle, the pathophysiological effects of aortic stiffening, and its association with cardiovascular events[85].

Age-related structural and functional changes in the large elastic arteries, leading to increased stiffness, are observed to precede the development of CVD. Furthermore, age-related vascular changes are accelerated in the presence of cardiovascular-related diseases. Aortic PWV is an independent predictor and is associated with higher risk of all-cause and cardiovascular-related mortality in hypertensive, diabetic, and end-stage kidney diseased individuals[86-88]. Additionally, aortic PWV adds predictive value, even when controlling for established cardiovascular risk factors, to the development of CVD[26-28].

Aortic PWV increases with advancing age even in normotensive adults free of clinical and occult disease[22, 23, 89]. Vaikevicius et al. found aortic PWV increases two-fold in a cross-sectional analysis of adults from 20 to 90 years of age[22]. When controlling for cardiovascular risk factors, a one standard deviation increase in aortic PWV is associated with a 48% increased risk of a first major cardiovascular event[76].

**Mechanisms of age-related arterial dysfunction**

Arterial function relies on the tight regulation of nitric oxide, oxidant, and inflammatory signaling pathways. Research supports that aging results in a dysregulation of these pathways through upregulation of pro-oxidant and

proinflammatory signaling that act in a feed-forward manner to further amplify one another and reduce nitric oxide bioavailability. This disruption of arterial homeostasis with age results in the development of vascular endothelial dysfunction and large elastic artery stiffness.

**Oxidative stress and aging**

Reactive oxygen species (ROS) are metabolites of oxygen with high reactivity. They are continuously generated as byproducts of normal aerobic metabolism but can be produced in higher amounts under stress and pathological conditions[90]. ROS play an important role at low levels in cell signaling as intracellular signaling molecules and paracrine messengers, and at acute high levels in antibacterial defenses. Reducing agents known as antioxidants normally inhibit ROS, but an imbalance of greater ROS relative to antioxidant defenses results in oxidative stress and leads to cellular dysfunction and damage[51]. ROS include radical (superoxide anion, hydroxyl radical, nitric oxide) and non-radical (hydrogen peroxide, peroxynitrite anion) molecules that oxidize macromolecules (lipids, nucleic acids, proteins)[90, 91]. Deleterious oxidation modifications include lipid peroxidation, nuclear and mitochondrial DNA mutations and deletions, amino acid alterations resulting in modification of protein activity, protein aggregation, and alterations in transcription factor activation[90]. ROS sources include the mitochondria and enzymatic systems (NADPH oxidase, xanthine oxidase, uncoupled NOS). Cross talk between these systems can result in a feed-forward production of greater amounts of ROS[92]. Antioxidants function to balance aerobic cellular ROS production and include enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (reduced glutathione, vitamin C and E, flavonoids,

alpha-lipoic acid, uric acid, ubiquinone) compounds[51].

***Endothelial function and oxidative stress.*** With aging, oxidative stress occurs in the vasculature primarily due to increased superoxide production by the mitochondria, NADPH oxidase, and eNOS uncoupling. Superoxide anion, a one-electron reduction of oxygen, has a short half-life of 30 milliseconds, and is therefore limited to its area of production[91]. Mitochondria are considered the predominant intracellular site of superoxide production, which is generated from electrons leaking from the mitochondrial electron transport chain. A negative correlation of aortic mitochondria superoxide production and EDD has been reported in older mice[93].

Acute *ex vitro* carotid incubation with the superoxide dismutase (SOD) mimetic TEMPOL restores age-related EDD in older male mice to levels of young mice[43, 94, 95]. Furthermore, 3 weeks of TEMPOL supplementation in the drinking water of older male mice restored NO-mediated EDD, aortic superoxide production, nitrotyrosine (a marker of peroxynitrite damage), NADPH oxidase, and inflammatory cytokine protein levels in older but not younger mice[43]. In humans, acute infusion of the direct scavenger of superoxide vitamin C restores age-impaired EDD in older men and postmenopausal women to levels of young adults[10, 96, 97].

Superoxide can react with NO at a higher rate than SOD to form peroxynitrite, thereby reducing NO bioavailability[91, 98]. Peroxynitrite can uncouple eNOS, resulting in superoxide production rather than NO. eNOS can also become uncoupled when there are reduced amounts of the essential cofactor, $BH_4$. In turn, $BH_4$ is highly susceptible to oxidative degradation by superoxide or peroxynitrite, quenching NO activity[91, 99]. Peroxynitrite performs post-translational nitration of tyrosine residues (nitrotyrosine),

upregulates the proinflammatory NOS isoform, inducible NOS (iNOS) expression, oxidizes $BH_4$, and oxidizes eNOS, resulting in further ROS production. Nitrotyrosine is higher in aortas of older male mice[43, 94, 95] and biopsied endothelial cells of older men[7]. In addition, endothelial cell nitrotyrosine protein levels are inversely related to brachial artery FMD in older men[7].

While reduced NO bioavailability is consistently observed with advancing age, preclinical models report inconsistent (increased, unchanged, decreased) changes in eNOS expression/activation with age[16]. However, in older healthy men, eNOS protein expression and phosphorylation at Ser1177 is paradoxically higher compared to young healthy men[100]. These data suggest that eNOS expression and activity may be upregulated in older adults to compensate for reduced NO bioavailability, and if uncoupled, eNOS may act as a source of superoxide production, not only reducing NO production but also increasing oxidative stress[16]. Most research supports that increases in superoxide levels with age are largely attributable to increased superoxide production.

However, whether superoxide removal is changed due to changes in antioxidant levels with age remains inconsistent. SOD has three isoforms based on location, extracellular (EC)SOD, copper-zinc (CuZn)SOD, and manganese (Mn)SOD located extracellularly, in the cytoplasm, and in the mitochondria, respectively. All isoforms convert superoxide into hydrogen peroxide and oxygen. CuZnSOD accounts for 50-80%, MnSOD for 2-12% and ECSOD for the remaining activity of overall SOD. ECSOD is produced most commonly by vascular smooth muscle cells and is thought to protect NO diffusing from the endothelium to the smooth muscle[98]. SOD expression is

unchanged or reduced in older humans and mice that have age-related impairments in EDD[16, 43, 94, 95]. MnSOD and ECSOD deficient mice demonstrate impaired NO-mediated EDD and higher superoxide production[93, 101].

***Stiffness and oxidative stress.*** Endothelium-derived factors regulate vascular smooth muscle tone and contribute to arterial stiffness. Removal of the endothelium in animals has shown to alter large artery stiffness. Some studies in diseased and healthy adults have shown an inverse correlation between endothelial function and large elastic artery stiffness, where NO donors in animals reduce aortic PWV [82]. This suggests that NO may directly modulate stiffness and superoxide-mediated reductions in NO indirectly affect vascular smooth muscle tone. Endothelium-derived NO is not only a crucial vasodilator, but also directly inhibits the potent endothelium-derived vasoconstrictor, ET-1, while ET-1 also directly inhibits NO[102]. Acute ET-1 vasoconstriction FBF response is blunted in older healthy men compared to young[103]. Older men have higher ET-1 endothelial cell protein expression compared to young and ET-1 expression is positively related to nitrotyrosine endothelial cell protein expression and inversely related to EDD[100]. Acute ET-1 inhibition restores EDD in older male mice[100] and improves EDD in older men[103].

Preclinical studies suggest that superoxide production is associated with changes in the structural properties of arteries with age, including increased arterial collagen deposition and AGEs accumulation, and reductions in elastin content[31, 43, 104-106]. In aortic segments from young mice, incubation with the superoxide generator pyrogallol induces aging-like increases in AGEs, and direct AGE incubation induces stiffening[106]. In addition, 3 weeks of TEMPOL administration normalizes age-related

superoxide production, collagen levels, and aortic PWV in older mice to that of young mice[105].

Circulating markers of oxidative stress and endothelial cell superoxide production are reported to be independently predictive and related to arterial stiffness, respectively, in adults[107, 108]. Acute supraphysiological vitamin C infusion had no effect on arterial stiffness in older healthy men[97] but improved age-impaired arterial stiffness in postmenopausal women[109], suggesting sex-related differences in the mechanisms contributing to arterial stiffness.

Taken together, these observations indicate that advancing age leads to the development of superoxide-mediated oxidative stress in the vasculature, while changes in antioxidant defenses with age remain inconsistent.

**Inflammation and aging**

Aging results in the suppression of adaptive immunity and the upregulation of innate immune signaling, leading to a phenotype of chronic low-grade inflammation known as “inflammaging”[19]. Aging is associated with increases in circulating inflammatory markers including C-reactive protein (CRP), tumor necrosis factor (TNF)-α, and interleukin (IL)-6, and these inflammatory markers predict CVD onset[100, 110, 111]. Higher levels of inflammatory proteins are reported in the aorta of older mice and biopsied endothelial cells of older adults compared to young adults[70, 71, 95, 100, 112].

A central mediator of age-associated increases in vascular inflammation is the transcription factor nuclear factor kappa-light chain-enhancer of activated B cells (NF-kB)[7, 70]. NF-kB is activated by a wide variety of stimuli including inflammatory cytokines, ROS, lipids and mechanical forces and activation results in a proinflammatory,

proadhesion, and pro-oxidant gene transcription, including cytokines and NADPH oxidase[100]. This further increases macromolecule damage and superoxide production, perpetuating a vicious cycle of increased ROS and inflammation, further unbalancing vascular homeostasis.

***Endothelial function and inflammation.*** Older mice with age-related impairment of NO-mediated EDD have higher aortic phosphorylation of NF-kB and proinflammatory cytokine (IL-6, TNF-α, interferon [IFN]-γ, IL-1β) protein expression than young mice[71, 95, 113]. Older mice that consumed salicylate, an aspirin-derived compound that inhibits the NF-kB activator IKKβ for 5 days had reversed age-associated impairment of NO-mediated EDD and reversed NF-kB and proinflammatory protein levels. TEMPOL incubation had no effect on EDD in salicylate-treated older mice, and salicylate reversed age-related NADPH oxidase p47 subunit and MnSOD protein levels[113].

Older adults with age-related impaired EDD have been reported to have higher NF-kB protein expression in biopsied endothelial cells[7, 70]. NF-kB p65 subunit protein expression was correlated with nitrotyrosine protein levels in older men. Older men also had higher NADPH oxidase p47 subunit endothelial cell protein levels compared to young men[7]. Salsalate (non-acetylated salicylate) supplementation for 3 days reduced age-associated NF-kB p65 subunit endothelial cell protein expression by 25% and improved brachial artery FMD in older adults. Acute infusion of vitamin C improvements in brachial artery FMD were abolished after the salsalate intervention and salsalate reduced nitrotyrosine by 25% and NADPH-oxidase p47phox subunit by 30%[20].

Vascular proinflammatory (IL-6, TNF-α, monocyte chemoattractant protein

[MCP]-1) endothelial cell protein levels[70] and circulating proinflammatory markers (CRP, IL-6) are higher in older adults with age-related impaired EDD[70, 71, 114]. However, circulating inflammatory markers did not relate to vascular inflammatory endothelial cell protein expression in older adults, suggesting that the circulating inflammatory state may not be a direct measure of the current vascular inflammatory state[70].

***Stiffness and inflammation.*** Inflammatory signaling stimulates oxidant enzyme systems to produce ROS[115] and ROS also promote a proinflammatory cascade[116]. This in turn reduces NO bioavailability, potentially altering vascular tone and accelerating damage and cross-linking of structural proteins.

Older male mice are reported to have ~70% greater aortic PWV associated with greater proinflammatory markers (IL-1β, IL-6, IFN-γ, TNF-α) and oxidative stress (higher aortic nitrotyrosine, NADPH oxidase p67 subunit, and superoxide production and reduced SOD) compared to young mice[95]. Acute induction of inflammation (via vaccination) increases CRP, IL-6, and matrix metalloproteinase (MMP)-9, as well as arterial stiffness (aortic PWV) in healthy young adults. Pre-treatment with an anti-inflammatory (aspirin) inhibits the vaccination-induced proinflammatory increases in arterial stiffness[117]. Inflammatory (TNF-α) inhibition by Etanercept administration for 12 weeks improves aortic PWV, brachial artery FMD, and CRP in all-aged rheumatoid arthritis patients[118]. Older sedentary adults with ~37% higher aortic PWV compared to young had improved arterial stiffness with 4 days of salsalate treatment in the absence of changing blood pressure. Improvement was inversely correlated with baseline aortic PWV[119].

In summary, these observations indicate that advancing age leads to the

development of chronic low-grade inflammation in the vasculature, with the pro-inflammatory transcription factor, NF-kB, acting as a central mediator.

**Therapeutic potential of curcumin**

Although healthy lifestyle behaviors appear to be the best method for protecting against vascular aging, many older adults do not meet the minimal recommended guidelines for exercise or consume a healthy diet. As a result, identifying alternative prevention strategies and interventions that improve age-related EDD and arterial stiffness that are both effective and safe in humans is of utmost importance.

Curcumin, a naturally occurring phenol, is found in the rhizome of the plant *Curcuma Longa*, a member of the ginger family. Curcumin is considered the major active component of turmeric and gives the Indian spice its yellowish color[120]. Curcumin is commonly used in yellow mustard, pickles, and sauces due to its color, flavor, and antioxidant stabilizing abilities. It has been used to flavor foods and treat ailments related to the skin, liver, gastrointestinal tract, and the common cold for over 4000 years, and has been generally recognized as safe by the Food and Drug Administration[120]. Studies performed in rodents and humans using standard toxicology protocols have shown no toxic effects of curcumin and indicate that curcumin is safe at doses as high as 8 grams/day[121-123]. According to the 1974 Food and Agriculture Organization of the United Nations and World Health Organization report, curcumin consumption in adults in India is ~60-100 mg/day.

Curcumin has been shown to exhibit pleiotropic effects by having a diverse range of molecular targets including transcription factors, genes, growth factors, inflammatory cytokines, enzymes, and adhesion molecules[124]. Curcumin may directly or indirectly

modulate its molecular targets with more than 30 different proteins having been found to directly interact with curcumin (superoxide, nuclear factor erythroid-derived 2-like 2 [Nrf2; antioxidant transcription factor], NF-kB, intercellular adhesion molecule [ICAM]-1, cyclooxygenase [COX]-2)[120, 124]. Curcumin has been reported to have anticoagulant, antioxidant, anti-inflammatory, antibacterial, and anticarcinogenic effects[120]. Curcumin's potential beneficial effects have been assessed in pathologies such as neurodegenerative diseases, cancer, rheumatoid arthritis, diabetes, and CVD[120].

Although the exact mechanisms underlying these beneficial outcomes are incompletely understood, curcumin has been reported to attenuate oxidative stress and inflammation both *in vitro* and *in vivo*. Cell culture models demonstrate that curcumin protects against stressors, including TNF-α or high glucose stimulation, by inhibiting proinflammatory transcription factor (NF-kB) activation, proinflammatory protein (IL-1β, IL-6, MCP-1, COX-2) production, ROS production, and cell adhesion molecule (ICAM-1) expression, and activating antioxidant (Nrf2) and vasodilator pathways (eNOS phosphorylation and NO production)[34-38, 125].

Most research with curcumin supplementation has been performed in diseased animal models. Curcumin has been shown to protect against or reduce the deleterious effects of atherosclerosis, diabetes, hypertension, and ischemia[36, 39-42, 126-132]. The protective effects of curcumin in these and other diseased or stressor models were mitigated through a reduction in inflammatory (NF-kB, TNF-α, IL-6, MCP-1, ICAM-1) and ROS (superoxide production, NADPH oxidase) signaling, and increased eNOS phosphorylation and antioxidant (Nrf2, SOD) defenses[36, 39, 40, 42, 127, 128, 130, 131, 133-136]. Furthermore, curcumin protected against or improved *ex vivo* EDD in these pathological

animal models[41, 129, 132, 133, 135, 137 40, 42]. Tetrahydrocurcumin, a metabolite of curcumin, supplementation at 13 months of age has been reported to extend longevity (~11%) in male mice[138].

Curcumin supplementation research in healthy young and diseased human populations has reported reduced inflammation (NF-kB, COX-2, soluble-ICAM) and oxidative stress, and increased circulating NO[120, 139, 140].

Research assessing the effects of curcumin on primary aging has been limited. Recent preclinical findings from my laboratory demonstrate 4 weeks of curcumin supplementation in chow restored NO-mediated carotid *ex vivo* EDD in older mice to levels of young mice. Improvements in EDD were associated with reduced oxidative stress as acute *ex vivo* administration of the superoxide dismutase mimetic TEMPOL, restored EDD in older non-supplemented mice but had no effect on EDD in older curcumin-supplemented mice and young mice. In addition, curcumin ameliorated age-related large elastic stiffness (aortic PWV) and these improvements were mitigated by reduced collagen and AGE protein expression in older (26-28 months) male mice to levels of young (4-6 months), with no effect in young mice. Curcumin reversed age-related aortic nitrotyrosine protein expression, superoxide production, NADPH oxidase p67 subunit protein expression, MnSOD, and inflammatory cytokines (IL-6 and TNF-α)[43].

In humans, limited research has been performed assessing the effects of curcumin on age-related arterial dysfunction. One study has reported that 8 weeks of curcumin supplementation improves brachial artery FMD and carotid compliance (vessels' ability to change volume relative to a change in pressure) in healthy middle-

aged and older Japanese postmenopausal women[44, 45]. Additionally, 8 weeks of curcumin supplementation improved brachial artery FMD in young adults[141].

In summary, curcumin restores and improves EDD in diseased and limited healthy animal models by restoring NO bioavailability and reducing oxidative stress and inflammation. Initial research in healthy older women for curcumin to improve age-related arterial dysfunction is promising but incomplete.

**Conclusions and future directions**

Vascular aging is the major risk factor for CVD. With the aging of our population and the estimated increase in age-related medical costs, there is a pressing need for preventive strategies and interventions that may reduce the risk of CVD. Aging, once thought to be inevitable, is now recognized as a modifiable risk factor. Interventions that target age-induced oxidative stress and inflammation hold promise to prevent or reduce arterial dysfunction with age and therefore prevent the development of CVD. Curcumin, a naturally occurring and safe phenol, has been shown to restore arterial function in older mice and limited, but promising, research in older women shows similar beneficial effects. However, more research is needed to assess the arterial protective effects of curcumin in both older men and women, and to assess the mechanisms involved therein.

# Chapter IV

## Curcumin supplementation improves micro- and macrovascular endothelial function in healthy middle-aged and older adults by increasing nitric oxide bioavailability and reducing oxidative stress

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**Abstract**

I hypothesized that curcumin supplementation would improve micro- and macrovascular endothelial function in healthy middle-aged and older adults. Thirty-nine healthy men and postmenopausal women (45-74 yrs) were randomized to 12 weeks of oral curcumin (2000 mg/day Longvida®; n=20) or placebo (n=19) supplementation. Forearm blood flow response to acetylcholine infusions ($FBF_{ACh}$) (resistance artery endothelial function) increased 37% following curcumin supplementation (107±13 vs. 84±11 AUC at baseline, P=0.03), but not placebo (P=0.2). Curcumin treatment augmented the acute reduction in $FBF_{ACh}$ induced by the nitric oxide synthase inhibitor *NG* monomethyl-L-arginine (L-NMMA; P=0.001), and abolished the acute increase in $FBF_{ACh}$ to the antioxidant vitamin C (P=0.3), whereas placebo had no effect. Similarly, brachial artery flow-mediated dilation (conduit artery endothelial function) increased 36% in the curcumin group (5.7±0.4 vs. 4.4±0.4% at baseline, P=0.001), with no change in placebo (P=0.1). Neither curcumin nor placebo influenced circulating biomarkers of oxidative stress and inflammation (all P>0.1). In healthy middle-aged and older adults, 12 weeks of curcumin supplementation improves microvascular endothelial function by increasing vascular nitric oxide bioavailability and reducing oxidative stress, while also improving macrovascular (conduit artery) endothelial function.

**Introduction**

Micro- (resistance artery) and macrovascular (conduit artery) endothelial function, as measured by endothelium-dependent dilation (EDD), decline with advancing age[5-11] and each is independently predictive of future risk of cardiovascular events and mortality[12-15]. Vascular endothelial dysfunction with age appears to develop

first in the microvasculature and subsequently in the macrovasculature[5, 9, 142]. A key mechanism mediating the development of age-related endothelial dysfunction is reduced bioavailability of the vascular protective and vasodilatory molecule nitric oxide[10, 16-18]. Decreased nitric oxide bioavailability with age is in part driven by the presence of oxidative stress, an increase in reactive oxygen species relative to antioxidant defenses, and chronic low-grade inflammation[8, 19-21]. With the number of older adults in the United States expected to double by the year 2050[46], interventions that improve age-related vascular endothelial dysfunction are needed to reduce the risk of CVD in this growing population.

Curcumin is a naturally occurring phenol found in the Indian spice turmeric. Curcumin has been reported to increase nitric oxide production and reduce oxidative stress and inflammation in cell and animal models of vascular-related disease [34-42], as well as healthy and diseased human populations[120, 139, 140]. In a recent preclinical study from my laboratory[43], it was demonstrated that 4 weeks of curcumin supplementation improved macrovascular endothelial function in older male mice to levels of young animals, mediated by an increase in nitric oxide bioavailability and a reduction in vascular oxidative stress. Taken together, these data suggest that curcumin supplementation holds promise as a treatment strategy for age-related arterial dysfunction.

The purpose of this study was to translate my laboratory's preclinical findings in older mice to healthy middle-aged and older adults. I hypothesized that curcumin supplementation would improve age-related vascular endothelial function in middle-aged and older men and postmenopausal women by increasing nitric oxide

bioavailability secondary to a reduction in oxidative stress, while also improving markers of systemic inflammation. To test this hypothesis, I performed a double-blind, parallel design, randomized study in which thirty-nine participants received curcumin (2000 mg/day Longvida® pill) or placebo supplementation for 12 weeks. Microvascular EDD in the absence and presence of intact nitric oxide production and oxidative stress, along with macrovascular EDD and circulating markers of oxidative stress and inflammation were measured at baseline (week 0), week 4 (macrovascular EDD only), and week 12.

## Methods

***Participants.*** Thirty-nine healthy men and postmenopausal women aged 45 to 74 years from Boulder County, Colorado and the surrounding areas were studied. All participants were non-smokers, sedentary or moderately physically active, and free of clinical diseases, including peripheral arterial disease (ankle-brachial index >0.90), as determined by medical history, physical examination, blood chemistries, and blood pressure and electrocardiogram at rest and during incremental treadmill exercise. All postmenopausal women were amenorrheic ≥1 year and postmenopausal women ≤56 years of age had a follicular stimulating hormone concentration ≥40 IU/L. Participants demonstrated age-related macrovascular endothelial dysfunction at screening, defined as brachial artery flow-mediated dilation (FMD) <7%. All procedures were reviewed and approved by the Institutional Review Board at the University of Colorado Boulder. The nature, risks, and benefits of all study procedures were explained to volunteers and their written informed consent was obtained before participation in the study. This study was registered on ClinicalTrials.gov (NCT01968564).

***Measurements.*** All measurements were performed at the University of Colorado Boulder Clinical Translational Research Center (CTRC) after a >12-hour fast (water allowed) from food, caffeine, and dietary supplements, and >24-hour refrainment from alcohol, physical activity, and prescription medications[143]. I, blinded, performed all primary data acquisition and analysis.

***Participant Characteristics.*** Body mass index and waist and hip circumferences were determined by anthropometry[144]. Percent body fat was measured using dual-energy X-ray absorptiometry (DEXA; GE Lunar Prodigy Advance). Arterial systolic and diastolic blood pressures were assessed in triplicate over the brachial artery at rest with a semi-automated device (Dinamap XL, Johnson & Johnson). Maximal oxygen consumption ($VO_2$ max) was measured during incremental treadmill exercise testing performed to exhaustion (Balke protocol) using open circuit spirometry, as previously described[145].

***Circulating Humoral Factors.*** All blood samples were drawn from an intravenous catheter at the cubital vein. The Colorado Clinical and Translational Sciences Institute CTRC Core Laboratory and Boulder Community Hospital Clinical Laboratory performed all blood assays, as previously described[146]. Fasting serum lipids were determined with standard assays. Fasting plasma glucose was measured by reflective spectrophotometry (Ortho Clinical Diagnostics) and fasting plasma insulin and serum adiponectin and leptin by radioimmunoassay (Millipore). Homeostasis model of insulin resistance (HOMA-IR) was calculated as [fasting plasma glucose (mg/dL) x fasting plasma insulin (μU/mL)]/405[147]. Serum follicular stimulating hormone was determined by chemiluminescence (Ortho Clinical Diagnostics). Serum high-sensitivity

C-reactive protein was measured by immunoturbidimetry (Beckman Coulter). Serum interleukin (IL)-6 and tumor necrosis factor (TNF)-α (R&D Systems), and plasma oxidized low-density lipoprotein (LDL) were assessed by ELISA (Mercodia). Serum TAS and whole blood glutathione peroxidase were measured by oxidative method (Randox Laboratories). Plasma epinephrine and norepinephrine were assessed by high performance liquid chromatography (BioRad) and plasma endothelin-1 (Peninsula Labs) by radioimmunoassay. Serum cortisol was determined by a one-step competitive assay (Beckman Coulter) and serum free fatty acids by enzymatic methods (Wako Chemicals USA).

***Curcumin Administration, Safety, and Tolerability.*** Participants were randomized to placebo or curcumin supplementation for 12 weeks in a double-blind manner using a blocked randomization scheme stratified for sex (male vs. female). Placebo or curcumin capsules (2000 mg/day Longvida® [~400 mg curcumin]) were taken once every morning while fasted. The Food and Drug Administration categorized curcumin as a supplement for the administration utilized in this study. Every two weeks of the intervention, participants underwent in-person check-in visits to exchange intervention capsules and to assess participant adherence by pill count. Tolerability and side effects were monitored at two-week check-in visits with adverse events documented by the CTRC staff and reported to the Institutional Review Board.

***Dietary Analysis.*** Participants were instructed by the CTRC Boulder registered dietitian to maintain their current caloric intake and avoid foods high in curcumin throughout the intervention. Average daily dietary intake was estimated by three-day diet records at baseline and week 12, and participants repeated the same diet the day

prior to all experimental visits. Diet records were analyzed by the CTRC Boulder registered dietitian using Nutrition Data System for Research.

***Microvascular Endothelial Function.*** Microvascular (resistance artery) EDD and endothelium-independent dilation were determined as FBF responses to incremental intrabrachial artery infusion of acetylcholine ($FBF_{ACh}$; 1, 2, 4, and 8 μg/100 mL forearm volume/min for 3.5-4 minutes per dose; Bausch and Lomb) and the nitric oxide donor sodium nitroprusside ($FBF_{SNP}$; 0.5, 1 and 2 μg/100 mL forearm volume/min for 3.5-4 minutes per dose; Marathon Pharmaceuticals LLC), respectively, using strain gauge venous occlusion plethysmography (A16 Arterial Inflow System, D.E. Hokanson) as previously described[6, 148]. To assess microvascular nitric oxide-mediated EDD, $FBF_{ACh}$ in the absence and presence of the nitric oxide synthase inhibitor, *NG* monomethyl-L-arginine (L-NMMA; 10 minute loading dose of 5 mg/minute at 2.5 mL/minute and maintenance dose of 1 mg/minute at 0.5 mL/minute; Bachem), was measured. Microvascular oxidative stress-mediated suppression of EDD was determined by $FBF_{ACh}$ in the absence and presence of the antioxidant vitamin C (10 minute loading dose of 25 mg/minute at 2.5 mL/minute and maintenance dose of 25 mg/minute at 0.5 mL/minute; Mylan Institutional LLC). FBF values are reported as individual dose responses and area under the dose-response curve (AUC).

***Macrovascular Endothelial Function.*** Macrovascular (conduit artery) EDD and endothelium-independent dilation were determined by brachial artery FMD (using a five-minute forearm cuff) and brachial artery diameter change following 0.4 mg sublingual nitroglycerin, respectively, using high-resolution ultrasonography (Toshiba Xario XG) as previously described[69, 143, 149]. Brachial artery FMD was measured at baseline, week 4,

and week 12, and brachial artery dilation to nitroglycerin at baseline and week 12. Brachial artery FMD and dilation to nitroglycerin are reported as percentage and absolute change from baseline diameter[143]. Brachial artery FMD shear rate was calculated as [8 x mean velocity (m/s)]/occlusion diameter (m)[143]. Brachial artery diameters and blood velocities were captured and analyzed by Vascular Research Tools 5.10.9 (Medical Imaging Applications).

***Data analysis.*** Statistical analyses were performed with IBM SPSS 23 and G*Power 3.1. Data normality was assessed with the Shapiro-Wilk test and non-normal variables were log base 10 transformed for statistical analysis. Outliers (≥3 standard deviations) were replaced with the group mean. An independent t-test was performed to assess group differences at baseline. A mixed-model ANOVA was performed to identify group (curcumin vs. placebo) by time (week 0, [4], and 12) interactions for all primary outcomes and clinical characteristics. To determine if there were sex-differences in the curcumin group after 12 weeks of supplementation, a mixed-model ANOVA was performed to identify any sex (men vs. women) by time interactions for all primary outcomes. In the case of significant interactions or significant overall effect of time, a paired t-test was performed for within-group comparisons. Sample size was estimated based on my laboratories' previous lifestyle intervention studies, using the primary outcome with the lowest effect size ($FBF_{ACh}$: 0.7) to detect significant group differences[150-153]. Data are expressed as mean±standard error (SE). Statistical significance was set at $\alpha<0.05$.

**Results**

***Participants.*** One hundred and eighteen participants were consented for the study. Fifty-seven individuals did not meet inclusion criteria. Seventeen individuals dropped from the study prior to randomization due to the time commitment (n=6), study restrictions (n=2), procedure invasiveness (n=1), or did not respond to scheduling requests (n=8). Twenty-one participants were randomized to the placebo group and twenty-three participants to the curcumin group. Two placebo group participants did not complete the study (excluded n=1, side effects: gastrointestinal discomfort; dropped n=1, time commitment). Two curcumin group participants did not complete the study (excluded n=1, side effects: dizziness; dropped n=1, non-study related medical concerns) and one participant was excluded from analysis due to a change in exercise status (**Figure 1**). Completed participants were of non-Hispanic Caucasian (n=32), non-Hispanic Asian (n=3), Hispanic Caucasian (n=2), non-Hispanic African American (n=1), or non-Hispanic American Indian/Alaskan (n=1) ethnicity.

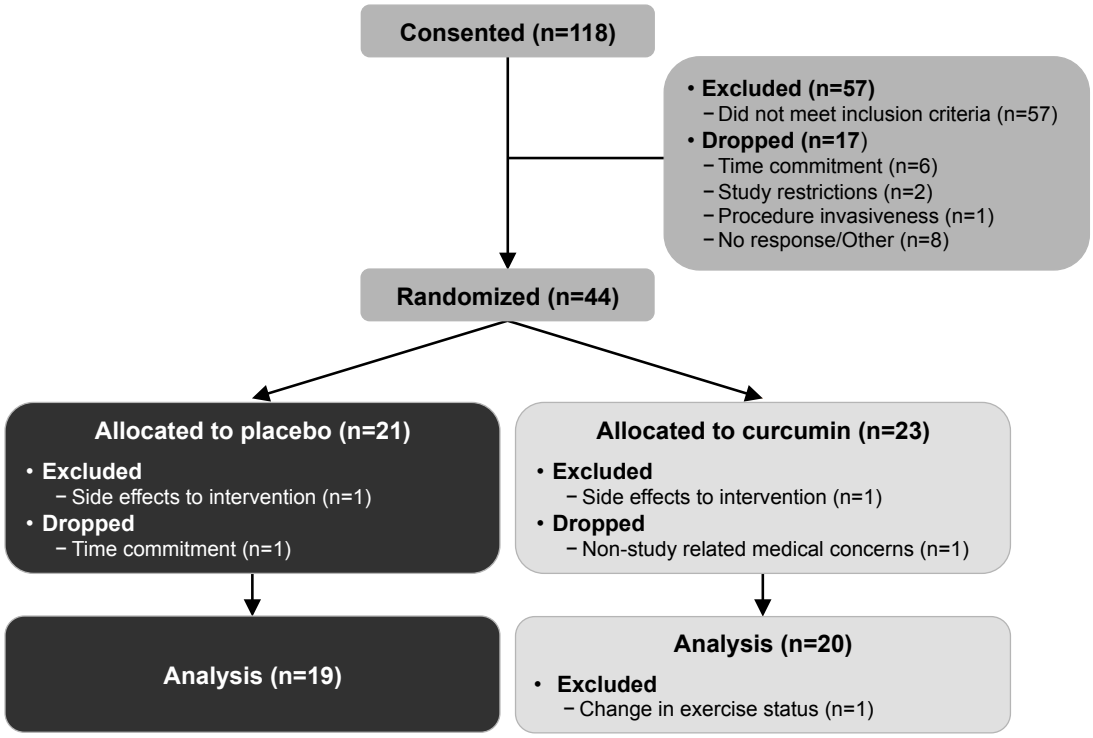


**Figure 1. Participant progress through study**

***Participant Characteristics.*** All participant characteristics (sex, age, body mass index, waist to hip ratio, body fat percent, blood pressure, heart rate, maximal oxygen consumption, lipids, glucose, C-reactive protein) were not different between the placebo and curcumin groups at baseline (all P>0.3) except for body mass, which was higher in the placebo group (P=0.03). Women were 11±2 years postmenopausal and 30% were previous hormone replacement users. No participant characteristics changed with time in either group (all within group and interaction comparisons P>0.05; **Table 1**).

**Table 1. Participant Characteristics**

| | Placebo | | Curcumin | |
|---|---|---|---|---|
| | Week 0 | Week 12 | Week 0 | Week 12 |
| N, men/women | 11/8 | -- | 10/10 | -- |
| Age, years | 61±2 | -- | 63±2 | -- |
| Body mass, kg | 76±3* | 75±3 | 68±2 | 68±3 |
| Body mass index, $kg/m^2$ | 25±1 | 25±1 | 24±1 | 24±1 |
| Waist to hip ratio, $U^L$ | 0.81±0.05 | 0.85±0.02 | 0.84±0.02 | 0.84±0.02 |
| Body fat, % | 27.9±2.0 | 27.8±2.0 | 30.1±1.9 | 30.2±1.9 |
| Systolic blood pressure, mmHg | 120±3 | 122±3 | 121±3 | 121±3 |
| Diastolic blood pressure, mmHg | 73±2 | 73±1 | 72±1 | 71±1 |
| Resting heart rate, $beats/min^L$ | 56±2 | 55±2 | 55±1 | 57±2 |
| $VO_2$ max, mL/kg/min | 33±1 | 33±1 | 31±1 | 31±1 |
| Total cholesterol, mg/dL | 177±6 | 173±6 | 175±8 | 174±6 |
| HDL-cholesterol, $mg/dL^L$ | 56±4 | 52±4 | 55±5 | 56±4 |
| LDL-cholesterol, mg/dL | 103±6 | 102±6 | 103±7 | 101±6 |
| Triglycerides, $mg/dL^L$ | 97±15 | 93±15 | 86±11 | 91±12 |
| Glucose, mg/dL | 84±2 | 85±2 | 85±2 | 87±2 |
| C-reactive protein, $mg/L^L$ | 0.96±0.26 | 1.15±0.34 | 0.81±0.14 | 0.72±0.12 |

*Data are mean±SE; $^L$Data log transformed for statistical analysis; *P=0.03 vs. curcumin week 0*

***Circulating Humoral Factors.*** Circulating humoral factors were assessed in a subset of participants (placebo n=11-12, curcumin n=13-14) and are presented in **Table 2**. All circulating blood factors (IL-6, TNF-α, oxidized LDL, TAS, glutathione peroxidase, epinephrine, norepinephrine, endothelin-1, cortisol, adiponectin, leptin, insulin, and

HOMA-IR) were not different between the placebo and curcumin groups at baseline (all P>0.1) except for free fatty acids, which were slightly lower in the placebo group (P=0.03). No circulating humoral factors changed with time in either group (all P>0.1).

**Table 2. Circulating Humoral Factors**

| | Placebo | | Curcumin | |
|---|---|---|---|---|
| | Week 0 | Week 12 | Week 0 | Week 12 |
| IL-6, pg/mL$^{L}$ | 0.84±0.19 | 1.64±0.48 | 0.90±0.12 | 1.16±0.20 |
| TNF-α, pg/mL$^{L}$ | 1.13±0.17 | 1.11±0.17 | 0.85±0.07 | 0.97±0.07 |
| Oxidized LDL, U/L | 30±2 | 33±2 | 33±3 | 33±3 |
| TAS, mmol/L | 1.48±0.05 | 1.47±0.06 | 1.44±0.05 | 1.45±0.04 |
| Glutathione peroxidase, U/L | 7497±555 | 7397±639 | 7591±497 | 7346±556 |
| Epinephrine, pg/mL | 31.72±5.47 | 28.36±4.05 | 24.85±2.66 | 25.85±3.79 |
| Norepinephrine, pg/mL | 286±23 | 315±40 | 303±30 | 323±48 |
| Endothelin-1, pg/mL | 5.29±0.29 | 4.83±0.17 | 5.90±0.47 | 5.42±0.33 |
| Cortisol, µg/mL | 9.42±0.73 | 9.33±0.81 | 8.29±0.69 | 8.86±0.95 |
| Free fatty acids, µmol/L | 433±27* | 415±66 | 546±39 | 486±31 |
| Adiponectin, µg/mL | 10.8±1.8 | 10.7±2.1 | 9.7±1.3 | 10.0±1.4 |
| Leptin, ng/mL$^{L}$ | 6.1±2.0 | 6.8±2.1 | 8.7±1.9 | 11.2±2.8 |
| Insulin, µU/mL$^{L}$ | 8±1 | 8±1 | 7±1 | 8±1 |
| HOMA-IR, U$^{L}$ | 1.6±0.2 | 1.6±0.2 | 1.5±0.2 | 1.6±0.2 |

*Data are mean±SE; $^{L}$Data log transformed for statistical analysis; *P=0.03 vs. curcumin week 0*

***Curcumin Safety and Tolerability.*** 72% of participants did not miss any intervention pills. Of the 11 participants who missed pills, 5 participants were in the curcumin group and missed a total of 4 to 12 pills throughout the intervention. No severe or unexpected adverse events occurred and the 2000 mg/day Longvida® formulation was well tolerated. Three curcumin group participants experienced "expected" adverse events, including dizziness (n=1), diarrhea (n=1), and gastrointestinal discomfort (n=1). The participant experiencing gastrointestinal discomfort dropped from the study. Four placebo group participants also experienced adverse events, including gastrointestinal discomfort (n=2), diarrhea (n=1), and nausea

(n=1). The placebo group participant who experienced gastrointestinal discomfort dropped from the study (**Table 3**).

**Table 3. Safety and Tolerability**

| | Placebo | Curcumin |
|---|---|---|
| Treatment-related adverse events, n | | |
| Diarrhea | 1 | 1 |
| Dizziness | 0 | 1 |
| Gastrointestinal discomfort | 2 | 1 |
| Nausea | 1 | 0 |
| Subjects with ≥ 1 adverse event, n | 0 | 0 |
| Dropouts, n | 1 | 1 |

***Dietary Analysis.*** Total daily energy, relative carbohydrate, and relative fat intake were not different between the placebo and curcumin groups at baseline (all P>0.3) except for daily relative protein intake, which was slightly higher in the placebo group (P=0.02). No dietary intake factors changed with time in the placebo and curcumin groups (all P>0.05; **Table 4**).

**Table 4. Dietary Intake**

| | Placebo | | Curcumin | |
|---|---|---|---|---|
| | Week 0 | Week 12 | Week 0 | Week 12 |
| Total daily energy (Kcal) | 1962±128 | 1945±83 | 2092±206 | 1974±198 |
| Daily relative carbohydrate (% of total Kcal) | 41±2 | 42±2 | 44±2 | 44±2 |
| Daily relative protein (% of total Kcal) | 20±1* | 19±1 | 16±1 | 18±1 |
| Daily relative fat (% of total Kcal) | 35±2 | 35±2 | 37±2 | 36±2 |

*Data are mean±SE; *P=0.02 vs. curcumin week 0*

***Microvascular Endothelial Function.*** $FBF_{ACh}$ was assessed as a measure of microvascular (resistance artery) endothelial function in a subset of participants (n=12 per group) due to difficulty placing intra-arterial lines in all participants both before and after the intervention period. $FBF_{ACh}$ AUC was not different between the placebo and curcumin groups at baseline (P=0.3). $FBF_{ACh}$ AUC had a group by time interaction between the placebo and curcumin supplementation groups (P=0.02). $FBF_{ACh}$ AUC

increased 37% after 12 weeks of curcumin supplementation (P=0.03), whereas there was no change with placebo (P=0.2; **Figure 2**). Individual $FBF_{ACh}$ AUC at baseline and week 12 for each group are presented in **Figure 3**. $FBF_{ACh}$ AUC was higher after 12 weeks of curcumin vs. baseline in 10 of the 12 participants treated with curcumin compared with only 2 of the 12 placebo group participants. No sex differences in $FBF_{ACh}$ AUC in the curcumin-supplemented group were observed (P=0.2; **Figure 4**). Microvascular endothelium-dependent dilation, a measure of vascular smooth muscle sensitivity to nitric oxide assessed as $FBF_{SNP}$, was not different between the placebo and curcumin groups at baseline (P=0.5) and did not change with time in either group (both P>0.4; **Figure 5**).

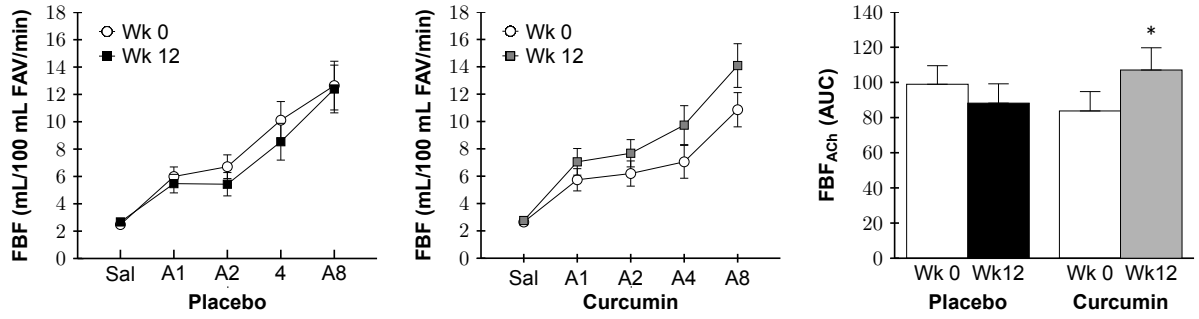


**Figure 2.** FBF in response to increasing doses (***left and middle***) and AUC (***right***) to acetylcholine at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; Group by time P=0.02; *P=0.03 vs. curcumin week 0*

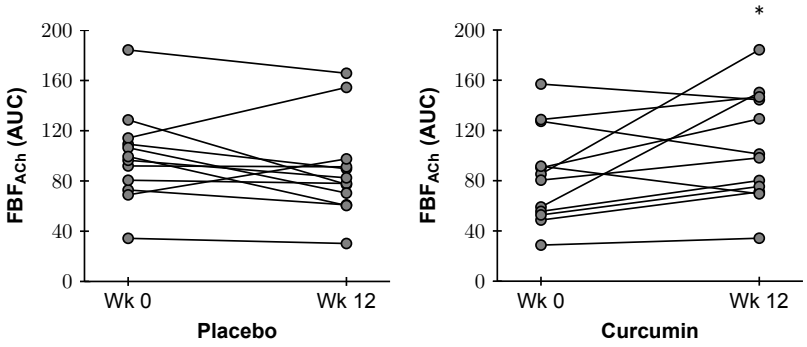


**Figure 3.** Individual $FBF_{ACh}$ AUC at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Group by time P=0.02; *P=0.03 vs. curcumin week 0*

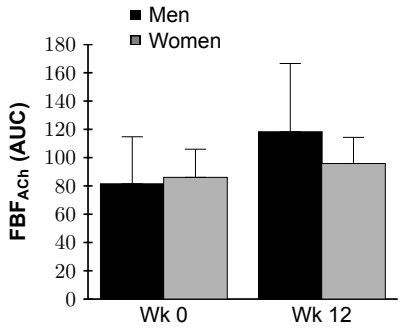


**Figure 4.** $FBF_{ACh}$ AUC for men and women at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; Sex by time P=0.2*

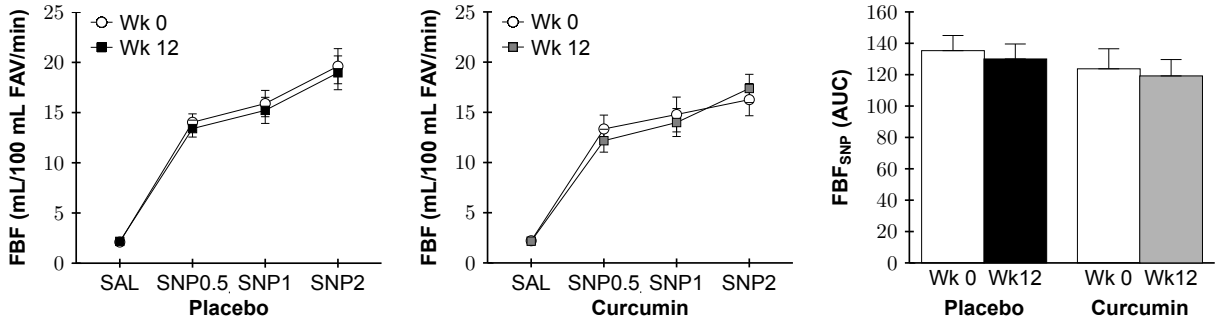


**Figure 5.** FBF in response to increasing doses (***left and middle***) and area AUC (***right***) to sodium nitroprusside at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; Group by time P=0.9*

***Microvascular Nitric Oxide-Mediated Endothelial Function.*** As shown in **Figure 6**, in the placebo group the reduction in $FBF_{ACh}$ AUC with co-infusion of the nitric oxide synthase inhibitor L-NMMA was similar at both week 0 and week 12 (both P=0.002), indicating a similar nitric oxide component of the vasodilation response to ACh at both time points. In contrast, in the curcumin-treated group the reduction in $FBF_{ACh}$ with L-NMMA was increased after 12 weeks of curcumin supplementation (P=0.001) compared with baseline (P=0.08). To further illustrate the contribution of nitric oxide to $FBF_{ACh}$, nitric oxide-dependent dilation was calculated as [($FBF_{ACh}$ with L-NMMA - $FBF_{ACh}$)/ $FBF_{ACh}$] x 100 and presented as positive values in **Figure 6**. There was a main effect of time (P=0.03), when the data were analyzed in this manner, which

was driven by an increase in nitric oxide-dependent dilation in the curcumin group (P=0.02), whereas no change was observed in the placebo group (P=0.6).

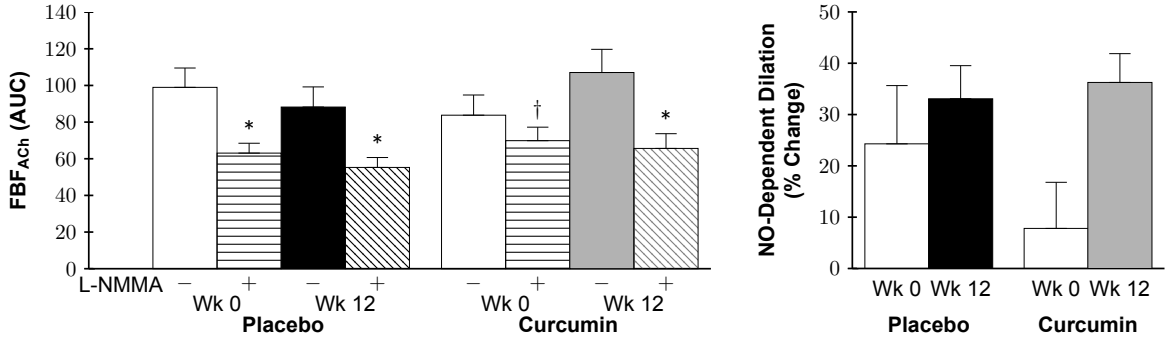


**Figure 6.** FBF$_{ACh}$ with (+) or without (-) L-NMMA (***left***) and nitric oxide (NO)-dependent dilation expressed as percent (***right***) at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; *P<0.02 vs. corresponding group week FBF$_{ACh}$; †P =0.08 vs. curcumin FBF$_{ACh}$ at week 0*

***Microvascular Oxidative Stress-Mediated Suppression of Endothelial Function.*** Co-infusion of the antioxidant vitamin C increased FBF$_{ACh}$ in both groups at baseline (both P<0.05), demonstrating a tonic oxidative stress-mediated suppression of microvascular function. After 12 weeks of curcumin supplementation, improvements in FBF$_{ACh}$ with vitamin C co-infusion were no longer observed (P=0.3), but remained present in the placebo group (P=0.03; **Figure 7**).

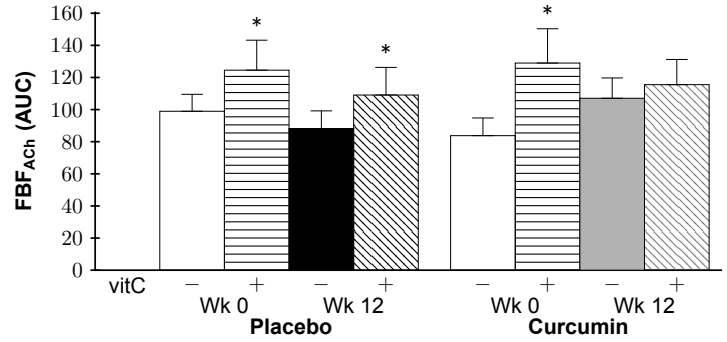


**Figure 7.** FBF$_{ACh}$ with (+) or without (-) vitamin C (vitC).
*Data are mean±SE; *P<0.05 vs. corresponding group week FBF$_{ACh}$*

***Macrovascular Endothelial Function.*** No group differences in brachial artery flow-mediated dilation (FMD), a measure of conduit artery EDD, or parameters were observed at baseline (all P>0.05). A group by time interaction was observed in brachial artery FMD percent (P=0.001) and absolute (P=0.001) change in which 12 weeks of curcumin supplementation increased brachial artery FMD 36% (P=0.001) with no changes in the placebo group (P=0.8; **Figure 8**; **Table 5**). Brachial artery FMD was higher after 12 weeks of curcumin vs. baseline in 17 of the 20 subjects treated with curcumin compared with only 7 of the 19 placebo group subjects. A strong trend for an improvement in FMD was observed at 4 weeks in the curcumin group (P=0.09), but not in the placebo group (P=0.2). Brachial artery dilation to nitroglycerin, a measure of macrovascular endothelium-independent dilation, was assessed in a subset of participants (placebo n=9, curcumin n=6) due to safety restrictions in administering nitroglycerin to individuals with low blood pressure or history of migraines. There were no significant effects of treatment on brachial artery dilation to nitroglycerin (P=0.8; **Figure 9**). In the curcumin-supplemented group, a sex by time interaction in brachial artery FMD was observed at 12 weeks (P=0.001), with significant improvements in both sexes (week 12 vs. baseline: men P=0.001, women P=0.01), but a greater magnitude of improvement in men compared with women (**Figure 10**).

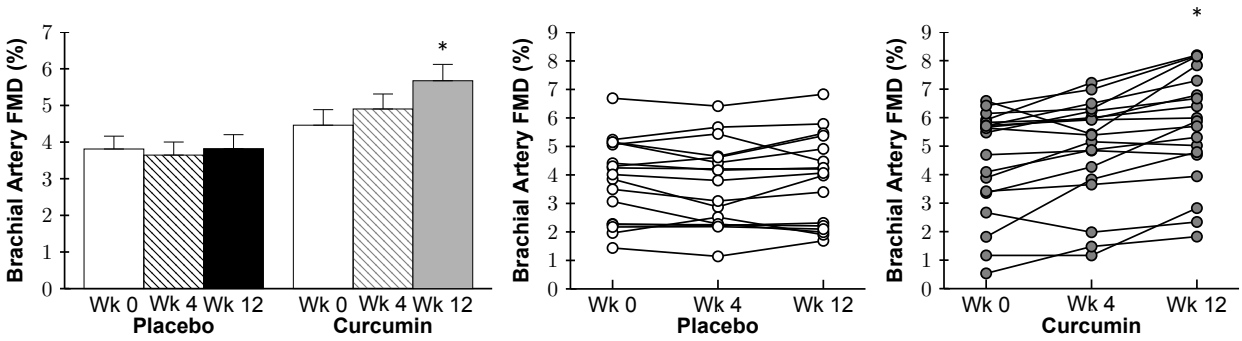


**Figure 8.** Brachial artery FMD expressed as percent change for group (***left***) and individuals (***middle, left***) at week 0 and after 4 and 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; Group by time P=0.001, *P=0.001 vs. curcumin week 0*

**Table 5. Brachial Artery Parameters**

| | Placebo | | | Curcumin | | |
|---|---|---|---|---|---|---|
| | Week 0 | Week 4 | Week 12 | Week 0 | Week 4 | Week 12 |
| Baseline diameter, mm | 3.77±0.18 | 3.76±0.18 | 3.76±0.17 | 3.40±0.14 | 3.42±0.15 | 3.38±0.15 |
| FMD absolute change, mm | 0.14±0.01 | 0.13±0.01 | 0.14±0.01 | 0.14±0.01 | 0.16±0.01 | 0.19±0.02* |
| Peak diameter, mm | 3.90±0.18 | 3.89±0.18 | 3.90±0.17 | 3.54±0.13 | 3.58±0.15 | 3.56±0.15 |
| Time to peak diameter, $s^{L}$ | 38±2 | 38±3 | 37±3 | 40±3 | 41±4 | 40±3 |
| FMD shear rate, $s^{-1L}$ | 1885±149 | 1816±190 | 1908±183 | 1977±166 | 2056±177 | 2150±170 |
| Nitroglycerin dilation, mm | 0.79±0.05 | -- | 0.77±0.04 | 0.77±0.10 | -- | 0.78±0.08 |

*Data are mean±SE; [L]Data log transformed for statistical analysis; *P<0.05 vs. curcumin week 0*

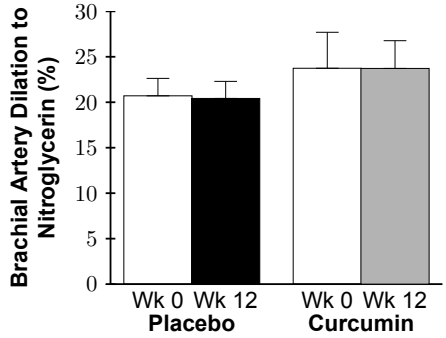


**Figure 9.** Brachial artery dilation to nitroglycerin expressed as percent change at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; Group by time P=0.8*

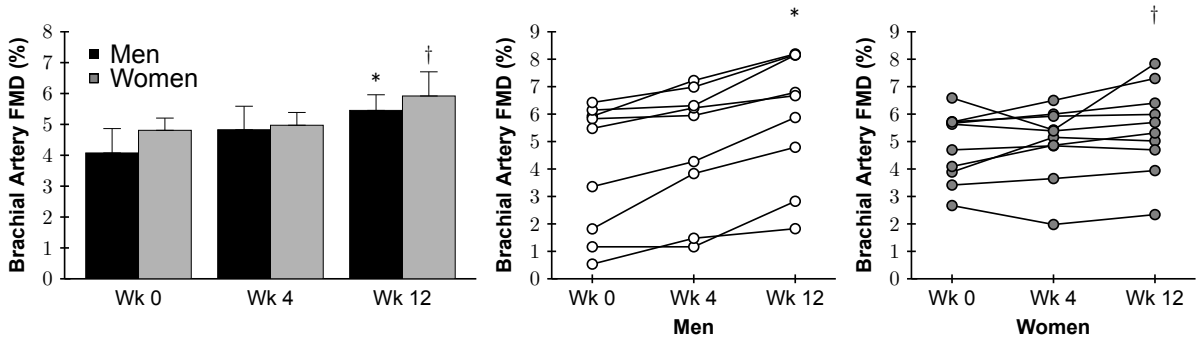


**Figure 10.** Brachial artery FMD expressed as percent change for men and women (***left***) and individual responses separated by sex (***middle and right***) at week 0 and after 4 and 12 weeks of curcumin supplementation.
*Data are mean±SE; Sex by time P=0.001; *P=0.001 vs. men week 0; †P=0.01 vs. women week 0*

## Discussion

This is the first study in humans to assess the beneficial effects of curcumin supplementation on age-related endothelial dysfunction within the micro- and macrovasculature and the mechanisms involved. The present findings demonstrate that 12 weeks of curcumin supplementation is safe and well tolerated and improves micro- and macrovascular endothelial function in healthy middle-aged and older adults. Improvements in both vascular beds were endothelium specific, as no changes in micro- or macrovascular endothelium-independent dilation were observed. As assessed in the microvasculature, the improvements in endothelial function were mediated by an increase in nitric oxide bioavailability and a reduction in vascular oxidative stress. In contrast, no changes in circulating biomarkers of oxidative stress and inflammation were observed after 12 weeks of curcumin supplementation.

***Curcumin Supplementation and Vascular Endothelial Function***. Micro- and macrovascular nitric oxide-mediated endothelial function declines with advancing age[5-11], with microvascular dysfunction believed to precede macrovascular impairments[5, 9, 142]. Measures of micro- and macrovascular endothelial function, $FBF_{ACh}$ and brachial

artery FMD, respectively, are each independently predictive of future risk of a cardiovascular event or mortality[12-15], although not necessarily correlated to one another[73]. When considered along with differences between conduit and resistance vessel structure and function[154], this suggests that each vascular bed may have relevance to different aspects of CVD and emphasizes the importance of assessing both the micro- and macrovasculature health.

*Microvascular Function, Nitric Oxide Bioavailability, and Oxidative Stress*. To my knowledge, no studies have assessed the effects of curcumin supplementation on microvascular endothelial function in the context of primary aging in preclinical models or humans. In diabetic and hypertensive rodent models, beneficial effects of curcumin on microvascular endothelial function has been reported in the heart, brain, and eye[155-157]. In the current study, I show that 12 weeks of oral curcumin supplementation improves microvascular endothelial function in healthy middle-aged and older men and postmenopausal women. Additionally, these improvements in $FBF_{ACh}$ were endothelium specific, as no changes in $FBF_{SNP}$ were observed. $FBF_{ACh}$ improvements after 12 weeks of curcumin supplementation were abolished by co-infusion with the nitric oxide synthase inhibitor L-NMMA, indicating that improvements in microvascular endothelial function were mediated by an increase in nitric oxide bioavailability.

In my laboratory's recent preclinical study[43], improvements in *ex vivo* carotid artery EDD with 12 weeks of curcumin supplementation in older mice were associated with reduced oxidative stress, as acute *ex vivo* administration of the superoxide dismutase mimetic, TEMPOL, restored EDD in older non-supplemented mice but had no effect on EDD in older curcumin-supplemented mice. Consistent with these

observations, in the present study 12 weeks of curcumin supplementation abolished the oxidative-stress mediated suppression of microvascular endothelial function, as evidenced by the lack of improvement of the $FBF_{ACh}$ response to co-infusion of the antioxidant vitamin C following, but not prior to, curcumin supplementation. Taken together, these observations indicate that a reduction in vascular oxidative stress was a key mechanism underlying improvements in nitric oxide-mediated microvascular endothelial dysfunction after 12 weeks of curcumin supplementation in my healthy middle-aged and older participants.

*Macrovascular Function*. Curcumin is reported to protect against or improve macrovascular endothelial dysfunction in animal models of cardio-metabolic disease, including diabetes, hypertension, and metabolic syndrome[40, 41, 129, 137]. However, research assessing the effects of curcumin on age-related macrovascular endothelial dysfunction has been limited.

A preclinical study performed by my laboratory[43] demonstrated that 4 weeks of curcumin-supplemented chow restored nitric oxide-mediated *ex vivo* carotid EDD in older (26-28 months) male mice to levels of young (4-6 months), with no effect in young mice. In humans, 8 weeks of curcumin supplementation improved brachial artery FMD in healthy Japanese postmenopausal women[44], similar to a new report in young adults[141].

In agreement with these findings, I found that 12 weeks of curcumin supplementation improved brachial artery FMD in healthy middle-aged and older men and postmenopausal women. This study is the first to measure macrovascular endothelium-independent dilation with curcumin supplementation in humans and

determine that these improvements in macrovascular EDD were endothelium specific, as no changes in brachial artery smooth muscle sensitivity to nitric oxide were observed. Additionally, I observed sex-differences in brachial artery FMD responsiveness to curcumin supplementation. Specifically, both men and women had significant improvements but the magnitude of improvement was greater in men compared with postmenopausal women. These latter observations are consistent with recent findings from my laboratory and others that changes in macrovascular endothelial function with interventions may be affected by sex[158-162]. Taken together, these data suggest that curcumin may be a promising therapeutic option to improve age-related vascular endothelial dysfunction in middle-aged and older adults.

***Curcumin Supplementation and Markers of Systemic Oxidative Stress and Inflammation.*** Cell culture and preclinical studies have demonstrated that curcumin has antioxidant and anti-inflammatory properties[34-42]. However, in the present study, no change was observed in circulating markers of oxidative stress (oxidized LDL, TAS, glutathione peroxidase) and inflammation (C-reactive protein, IL-6, TNF-α) with 12 weeks of curcumin supplementation. Although studies in humans evaluating the impact of curcumin supplementation on systemic markers of oxidative stress and inflammation are limited, 4 to 6 weeks of curcumin has been reported to reduce or have no effect on such circulating markers in healthy adults[140, 163]. Moreover, these circulating markers are not consistently altered in intervention studies that improve vascular endothelial function in healthy middle-aged and older adults[146, 153, 164]. The lack of change can be attributed to the relatively low levels of systemic oxidative stress and inflammation that, although typically are greater than levels in healthy young adults, are modest compared

with patients with chronic diseases, such as overt CVD[165, 166]. Importantly, recent studies suggest that circulating biomarkers may not be reflective of the local vascular endothelial state in healthy older adults[70, 74]. As such, the observed reduction in vascular oxidative stress, demonstrated by no improvement in EDD with vitamin C infusion following curcumin supplementation, provides the most relevant insight regarding the mechanism of action and endothelium-specific antioxidant effects of curcumin supplementation.

*Limitations.* To my knowledge there are no acute *in vivo* assessments of pro-inflammatory-mediated suppression of vascular endothelial function as there is for oxidative stress-mediated suppression of endothelial function using vitamin C infusion. The multiple-day administration of salsalate, a nuclear factor κB-inhibiting compound, is challenging and not feasible to administer before and after a chronic intervention. Therefore, I was unable to determine if a reduction in pro-inflammatory vascular signaling contributes to improvements in EDD with curcumin supplementation. Additionally, primarily healthy Caucasian men and women served as participants for this study. Therefore, it remains to be determined whether curcumin supplementation improves vascular endothelial function in other populations, including other ethnicities or individuals with greater arterial dysfunction compared with primary aging due to the presence of major/multiple risk factors for CVD (e.g., metabolic syndrome) or overt clinical CVD.

**Conclusion**

In healthy middle-aged and older men and postmenopausal women, 12 weeks of curcumin supplementation is well tolerated, and improves micro- and macrovascular

endothelial function, for at least the former, by increasing nitric oxide bioavailability and reducing vascular oxidative stress. However, curcumin supplementation did not reduce circulating biomarkers of oxidative stress or inflammation. These findings support curcumin supplementation as a promising nutraceutical-based treatment for improving nitric oxide-mediated vascular endothelial function and oxidative stress. As such, curcumin is a nutraceutical that may be helpful for maintaining a healthy vascular endothelium with aging, a key process in preventing the development of atherosclerosis and attendant arterial diseases. Additional studies are needed to fully elucidate the mechanisms underlying the beneficial effects of curcumin in humans and to examine the efficacy of curcumin supplementation in individuals with CVD or major risk factors for cardiovascular disorders.

# Chapter V

## 12 weeks of curcumin supplementation has no effect on regional or local large elastic artery stiffness in healthy middle-aged and older adults

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**Abstract**

Large elastic arteries stiffen with age, a key antecedent to the development of cardiovascular diseases. I hypothesized that curcumin would improve regional and local large elastic artery stiffness in healthy middle-aged and older adults. Thirty-nine healthy men and postmenopausal women (45-74 yrs) were randomized to 12 weeks of oral curcumin (2000 mg/day Longvida®; n=20) or placebo (n=19) supplementation. Large elastic artery stiffness was assessed regionally by aortic pulse wave velocity and locally by carotid artery compliance and carotid artery β-stiffness index. Neither curcumin nor placebo influenced measures of large elastic artery stiffness, including aortic pulse wave velocity, carotid artery compliance, or carotid artery β-stiffness index (all P>0.1). In healthy middle-aged and older adults, 12 weeks of curcumin supplementation has no effect on multiple indices of local and regional large elastic artery stiffness.

**Introduction**

Cardiovascular diseases (CVD) remain the leading cause of death in developed societies with aging a major risk factor[1, 2, 4]. Aging results in adverse changes to arteries including stiffening of the large elastic arteries (aorta and carotid arteries) [22-25]. Large elastic artery stiffness is an independent predictor of risk of cardiovascular-related mortality[26-28]. Stiffness of large elastic arteries is commonly assessed regionally by aortic pulse wave velocity (PWV) and locally by carotid artery compliance and carotid artery β-stiffness index[84, 167]. With the number of older adults expected to double by the year 2050[46], identification of preventative and intervention strategies to protect or improve large elastic artery stiffness is of the utmost biomedical importance.

Mechanisms contributing to the development of large elastic artery stiffness with age include changes in vascular smooth muscle tone and structural components of the arterial wall[4, 29, 30]. These changes with age are driven by reductions in nitric oxide bioavailability and increases in oxidative stress and inflammation[31-33].

Curcumin, the naturally occurring phenol in the Indian spice turmeric, has been reported to exert antioxidant and anti-inflammatory properties *in vivo* and *in vitro*[34-42]. In a recent preclinical study by my laboratory[43], 4 weeks of curcumin supplementation ameliorated aortic stiffening, as indicated by reductions in aortic PWV in older mice to that of young adult mice. Additionally, 8 weeks of curcumin supplementation improved carotid artery compliance and carotid artery β-stiffness index in healthy Japanese postmenopausal women[168]. Taken together, these data suggest that curcumin supplementation holds promise as a treatment strategy for age-related arterial dysfunction, but more research is needed, including assessing the efficacy of curcumin supplementation on regional and local large elastic artery stiffness in both healthy middle-aged and older men and postmenopausal women.

The purpose of this study was to translate my laboratory's preclinical findings in older mice to healthy middle-aged and older adults. I hypothesized that curcumin supplementation would improve age-related regional and local large elastic artery stiffness. To test this hypothesis, I performed a double-blind, parallel design, randomized study in which thirty-nine participants received curcumin (2000 mg/day Longvida® pill) or placebo supplementation for 12 weeks. Regional large elastic artery stiffness was assessed by aortic PWV and local large elastic artery stiffness by carotid artery compliance and carotid artery β-stiffness index at baseline (week 0) and week 12.

## Methods

***Participants.*** Thirty-nine healthy men and postmenopausal women aged 45 to 74 years from Boulder County, Colorado and the surrounding areas were studied. All participants were non-smokers, sedentary or moderately physically active, and free of clinical diseases, including peripheral arterial disease (ankle-brachial index >0.90), as determined by medical history, physical examination, blood chemistries, and blood pressure and electrocardiogram at rest and during incremental treadmill exercise. All postmenopausal women were amenorrheic ≥1 year and postmenopausal women ≤56 years of age had a follicular stimulating hormone concentration ≥40 IU/L. All procedures were reviewed and approved by the Institutional Review Board at the University of Colorado Boulder. The nature, risks, and benefits of all study procedures were explained to volunteers and their written informed consent was obtained before participation in the study. This study was registered on ClinicalTrials.gov (NCT01968564).

***Measurements.*** All measurements were performed at the University of Colorado Boulder Clinical Translational Research Center (CTRC) after a >12-hour fast (water allowed) from food, caffeine, and dietary supplements, and >24-hour refrainment from alcohol, physical activity, and prescription medications[143]. I, blinded, performed all primary data acquisition and analysis.

***Participant Characteristics.*** Body mass index and waist and hip circumferences were determined by anthropometry[144]. Percent body fat was measured using dual-energy X-ray absorptiometry (DEXA; GE Lunar Prodigy Advance). Arterial systolic and diastolic blood pressures were assessed in triplicate over the brachial artery at rest with

a semi-automated device (Dinamap XL, Johnson & Johnson). Maximal oxygen consumption ($VO_2$ max) was measured during incremental treadmill exercise testing performed to exhaustion (Balke protocol) using open circuit spirometry, as previously described[145].

***Circulating Humoral Factors.*** All blood samples were drawn from an intravenous catheter at the cubital vein. The Colorado Clinical and Translational Sciences Institute CTRC Core Laboratory and Boulder Community Hospital Clinical Laboratory performed all blood assays, as previously described[146]. Fasting serum lipids were determined with standard assays. Fasting plasma glucose was measured by reflective spectrophotometry (Ortho Clinical Diagnostics).

***Large Elastic Artery Stiffness.*** Large elastic artery stiffness was assessed regionally via aortic PWV and locally via carotid artery compliance and carotid artery β-stiffness index as previously described[85]. Briefly, central (aortic: carotid to femoral) and peripheral (carotid to radial) PWV was determined by applanation tonometry with simultaneous electrocardiogram gating of the R-wave to measure the time delay between the foot of the carotid and femoral or radial arterial pressure waves (Non-Invasive Hemodynamics Workstation, Cardiovascular Engineering Inc.). PWV was calculated as the distance between arterial sites divided by the arterial pressure wave transit time at each site.

Carotid artery compliance was assessed using ultrasonography (Toshiba Xario XG) to measure arterial diameter from end-systole to end-diastole while simultaneously measuring carotid arterial pressure changes via applanation tonometry as previously described[167]. Carotid artery compliance was calculated as (3.141592*((2*carotid

diastolic diameter)*(carotid systolic-diastolic diameter) + (carotid systolic-diastolic diameter)$^2$)/4*(carotid pulse pressure))[169]. Carotid artery β-stiffness index was calculated as (Ln(carotid systolic blood pressure/carotid diastolic blood pressure))/((carotid systolic diameter - carotid diastolic diameter)/carotid diastolic diameter)[170]. Carotid artery diameters were captured and analyzed by Vascular Research Tools 5.10.9 (Medical Imaging Applications).

***Data analysis.*** Statistical analyses were performed with IBM SPSS 23 and G*Power 3.1. Data normality was assessed with the Shapiro-Wilk test and non-normal variables were log base 10 transformed for statistical analysis. Outliers (≥3 standard deviations) were replaced with the group mean. An independent t-test was performed to assess group differences at baseline. A mixed-model ANOVA was performed to identify group (curcumin vs. placebo) by time (week 0 and 12) interactions for all primary outcomes and clinical characteristics. To determine if there were sex-differences in the curcumin group after 12 weeks of supplementation, a mixed-model ANOVA was performed to identify any sex (men vs. women) by time interactions for all primary outcomes. In the case of significant interactions or significant overall effect of time, a paired t-test was performed for within-group comparisons. Data are expressed as mean±standard error (SE). Statistical significance was set at $\alpha<0.05$.

## Results

***Participants.*** One hundred and eighteen participants were consented for the study. Fifty-seven individuals did not meet inclusion criteria. Seventeen individuals dropped from the study prior to randomization due to the time commitment (n=6), study restrictions (n=2), procedure invasiveness (n=1), or did not respond to scheduling

requests (n=8). Completed participants were of non-Hispanic Caucasian (n=32), non-Hispanic Asian (n=3), Hispanic Caucasian (n=2), non-Hispanic African American (n=1), or non-Hispanic American Indian/Alaskan (n=1) ethnicity.

***Participant Characteristics.*** All participant characteristics (sex, age, body mass index, waist to hip ratio, body fat percent, blood pressure, heart rate, maximal oxygen consumption, lipids, glucose) were not different between the placebo and curcumin groups at baseline (all P>0.3) except for body mass, which was slightly higher in the placebo group (P=0.03). Women were 11±2 years postmenopausal and 30% were previous hormone replacement users. No participant characteristics changed with time in either group (all within group and interaction comparisons P>0.05).

***Arterial Stiffness.*** There were no baseline group differences in aortic PWV, carotid artery compliance, carotid artery β-stiffness index (**Figure 1**), or other arterial stiffness parameters (all P>0.1; **Table 1**), except for change in carotid artery diameter, which was slighter lower in the placebo group (P=0.02). There was no group by time interaction for aortic PWV, carotid artery compliance, carotid artery β-stiffness index, or other arterial stiffness parameters (all P>0.2). No sex-differences in aortic PWV or carotid artery compliance and β-stiffness index in the curcumin-supplemented group were observed (both P>0.3; **Figure 2**).

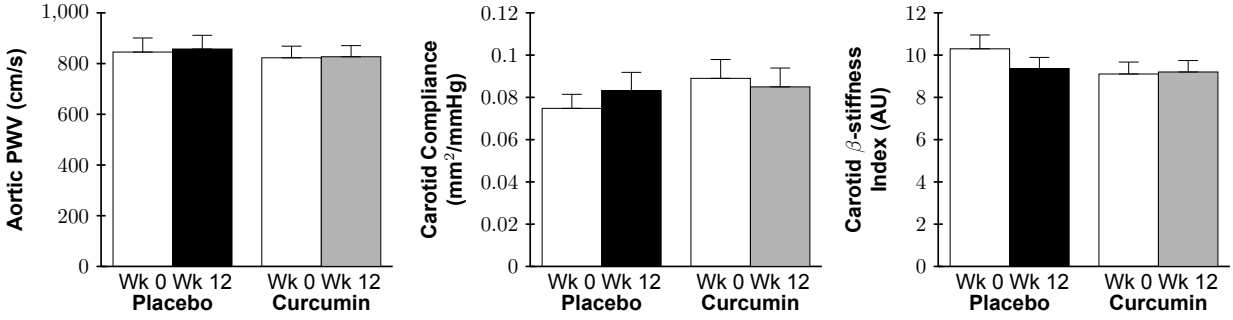


**Figure 1.** Aortic pulse wave velocity (***left***), carotid artery compliance (***middle***), and carotid β-stiffness index (***right***) at week 0 and after 12 weeks of placebo or curcumin supplementation.

*Data are mean±SE; Data log transformed for statistical analysis; All group by time P>0.2*

**Table 1. Arterial Stiffness Parameters**

| | Placebo | | Curcumin | |
|---|---|---|---|---|
| | Week 0 | Week 12 | Week 0 | Week 12 |
| Carotid systolic blood pressure, mmHg | 117±5 | 116±5 | 123±4 | 123±4 |
| Carotid pulse pressure, mmHg | 49±3 | 48±3 | 56±4 | 56±3 |
| Carotid artery diameter at end-diastole, mm$^{L}$ | 6.35±0.2 | 6.35±0.2 | 6.41±0.1 | 6.38±0.2 |
| Carotid change in diameter, mm | 0.35±0.02* | 0.36±0.02 | 0.45±0.03 | 0.44±0.03 |
| Carotid augmentation index, % | 16±3 | 16±3 | 17±2 | 17±2 |
| Carotid to radial PWV, cm/s | 991±26 | 989±34 | 945±34 | 930±47 |
| Radial augmentation index, % | -13±3 | -12±3 | -8±3 | -6±3 |
| Carotid IMT at end diastole, mm$^{L}$ | 0.56±0.01 | 0.56±0.01 | 0.60±0.02 | 0.60±0.02 |

*Data are mean±SE; IMT, intima-media thickness; $^{L}$Data log transformed for statistical analysis; *P=0.02 vs. curcumin week 0*

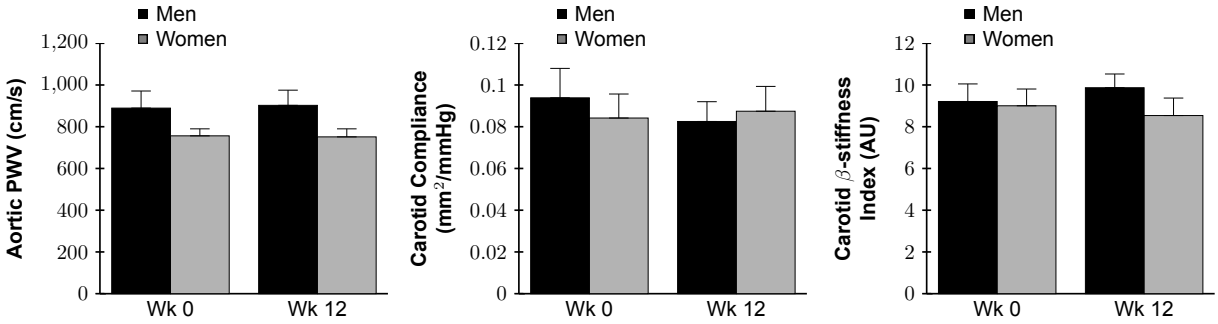


**Figure 2.** Aortic pulse wave velocity (***left***), carotid artery compliance (***middle***), and carotid β-stiffness index (***right***) for men and women at week 0 and after 12 weeks of placebo or curcumin supplementation.
*Data are mean±SE; All group by time P>0.2*

## Discussion

This is the first study in humans to assess the beneficial effects of curcumin supplementation on age-associated regional and local large elastic artery stiffness in healthy middle-aged and older men and postmenopausal women. In contrast to my hypothesis, no changes in regional or local large elastic artery stiffness were observed after 12 weeks of curcumin supplementation.

***Curcumin Supplementation and Large Elastic Artery Stiffness.*** Stiffening of

the large elastic arteries with age is attributed to a combination of functional and structural wall changes[4, 29, 30]. Arterial functional changes result primarily from increased vascular smooth muscle tone whereas structural wall changes are a consequence of extracellular matrix remodeling, including increased deposition of the load-bearing protein collagen and cross-linking proteins (advanced glycation end products), as well as a reduction and fragmentation of the elasticity conferring protein elastin[4, 29, 31].

A recent preclinical study performed by my laboratory[43] demonstrated that 4 weeks of curcumin supplementation reversed age-related large elastic artery stiffness (decreased aortic PWV) in mice and that these improvements were associated with reduced collagen and advanced glycation end products in the aorta. In healthy Japanese postmenopausal women, Akazawa et al.[168] reported that 8 weeks of curcumin supplementation improved carotid artery compliance. In contrast, in the present study no changes were observed in regional or local large elastic artery stiffness (aortic PWV or carotid artery compliance and carotid artery β-stiffness, respectively) after 12 weeks of curcumin supplementation in healthy middle-aged and older men and postmenopausal women. Analysis of potential sex differences indicated no improvements in postmenopausal women in the curcumin-supplemented group. Differences in carotid artery compliance outcomes between my study and that of Akazawa et al.[168] may be due to the reduction in carotid systolic blood pressure observed in the latter investigation, explaining the increase in carotid artery compliance but no significant change in carotid artery β-stiffness, a blood pressure independent index of arterial stiffness[170]. In the present study I did not observe any changes in carotid systolic blood pressure with 12 weeks of curcumin supplementation. Differences

in the subject populations studied—middle-aged and older men and women (mostly Caucasian) vs. postmenopausal Japanese women—also may have contributed.

In general, my findings here are consistent with a growing body of literature suggesting little or no effects of several weeks to months of nutraceutical-based treatment on large elastic artery stiffness in healthy middle-aged and older adults[97, 171-175]. Importantly, those studies showing improvements with nutraceutical interventions have only observed changes in indirect measures of stiffness (carotid artery compliance)[168, 176, 177] or implemented longer intervention periods of multiple years[178]. Differences in responsiveness between human and animal studies of nutrient-based interventions could be due to a number of factors, including differences in variation of genetic background, metabolism, length of intervention relative to lifespan, and environmental factors.

*Limitations.* Primarily healthy Caucasian men and women served as participants for this study. Therefore, it remains to be determined whether curcumin supplementation improves large elastic arterial stiffness in other populations, including other ethnicities or individuals with greater arterial dysfunction compared with primary aging due to the presence of major/multiple risk factors for CVD (e.g., metabolic syndrome) or overt clinical CVD.

**Conclusions**

Twelve weeks of curcumin supplementation had no effect on regional and local large elastic artery stiffness in healthy middle-aged and older men and postmenopausal women. Additional studies are needed to resolve discrepant findings on the effects of curcumin supplementation on large elastic artery stiffness in differing ethnic populations.

# Chapter VI

## Conclusions

The goal of this dissertation was to test the hypothesis that curcumin supplementation would improve micro- and macrovascular endothelial function in healthy middle-aged and older adults, and these improvements would be associated with increased nitric oxide bioavailability and reduced oxidative stress and inflammation. A secondary hypothesis was curcumin supplementation would improve regional and local large elastic artery stiffness in this same population.

As hypothesized, 12 weeks of curcumin supplementation increased micro- and macrovascular endothelial function. Improvements in both vascular beds were endothelium specific, as no changes in micro- or macrovascular endothelium-independent dilation were observed. As assessed in the microvasculature, the improvements in endothelial function were mediated by an increase in nitric oxide bioavailability and a reduction in vascular oxidative stress. In contrast, no changes in circulating biomarkers of oxidative stress and inflammation were observed after 12 weeks of curcumin supplementation.

In contrast to my secondary hypothesis, 12 weeks of curcumin supplementation had no effect on regional and local large elastic artery stiffness in healthy middle-aged and older men and postmenopausal women.

Taken together, these results indicate that curcumin supplementation, while having heterogeneous effects on the vasculature, improves vascular endothelial

function, a key antecedent to CVD, and therefore may be a promising prevention of the development of CVD in healthy middle-aged and older adults.

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