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IMPACT OF ACUTE INCREASES IN RETROGRADE SHEAR RATE ON MICROVASCULAR FUNCTION

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IMPACT OF ACUTE INCREASES IN RETROGRADE SHEAR RATE ON MICROVASCULAR FUNCTION

A THESIS APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

BY

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To my mom, wherever she is.

To my dad, my number one fan and idol.

Abuela Concha and Tita Rosa, who taught me the values of life.

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Abstract

Background: Research had well examined the influence of different levels of retrograde shear stress on macrovascular endothelial function. Data showed a negative impact on flow-mediated dilation (FMD), leading to macrovascular endothelial dysfunction and pro-atherosclerotic mechanisms. However, the impact of retrograde shear on microvascular function, measured in terms of microvascular reactivity, was missing in scientific literature.

Primary Aim: Determine the effect of acute changes in retrograde shear rate on microvascular function in healthy, young humans as determined via the post-occlusive NIRS response.

Methods: 17 healthy subjects participated in this study. Microvascular reactivity, via NIRS derived measurements of tissue oxygenation characteristics, was assessed in each participant before and immediately after exposure to a 30 min retrograde shear treatment. Retrograde shear treatment was achieved via a blood pressure cuff placed below the knee inflated to the target pressure of 75 mmHg. Only one leg of the subject was exposed to the shear stress treatment, with the contralateral leg serving as a control leg, which allowed comparisons between legs.

Results: In the treatment leg, the application of the external cuff resulted in significant increases in retrograde shear stress and an increase in the oscillatory shear index, not observed in the control leg. After acute exposure of the increased retrograde shear stress, the NIRS showed a significantly higher resting TSI% post-intervention in the treatment leg, as well as increases in [Hb]total and [HbO2] resaturation rates and peak TSI%, which were not present in the control leg.

Conclusions: The hypothesis that retrograde shear treatment would affect negatively microvascular function by decreasing the rate of resaturation, time-to-peak resaturation, and peak saturation, was rejected. The data however suggests that our intervention in the treatment leg had a positive impact on NIRS derived parameters of microvascular reactivity.

Chapter 1: Introduction

Hemodynamic shear stress within the cardiovascular system arises from the friction between blood and the endothelial cells lining the vasculature and is an important determinant of vascular health [1] . Throughout the cardiac cycle, the shear stress pattern within the peripheral vasculature can change such that a large anterograde shear occurs during systole and a varying degree of retrograde shear occurs during diastole [2]. Anterograde shear refers to shear stress caused by the directional flow of blood away from the heart distally to the periphery, while retrograde shear is caused by the reversal of flow back towards the heart. During periods of high vascular resistance, particularly due to low nitric oxide bioavailability [3], retrograde shear is increased and an oscillatory flow pattern is achieved across the entire cardiac cycle (i.e., high anterograde and retrograde flow).

The endothelial cells within the peripheral vasculature are highly sensitive to changes in hemodynamic shear stress, such that periods of increased retrograde and oscillatory shear acutely attenuate endothelial function and promote a pro-atherogenic (pro-atherosclerosis) endothelial cell phenotype [1]. In vitro studies using cell culture and isolated perfused arteries has established the casual link between this type of disturbed shear pattern and adverse endothelial adaptations. Chappell et al. (1988) studied human umbilical vein endothelial cells (HUVECs) in vitro and showed that oscillatory flow promoted adhesion of endothelial leukocyte (in special monocytes) expression via increased levels of three adhesion molecules: vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin [4]. This finding partially explains why atherosclerotic lesions are present in arterial flow

dividers, where oscillatory flow is exhibited. Chappel et al. (1998) also pointed out that upregulation of atherosclerosis mechanisms are related to changes in oxidative stress, such as reactive oxygen species (ROS) and NADPH oxidase [4]. In this same line of free radicals and ROS, McNally et al. (2003) found in bovine aortic endothelial cells (BAECs) and murine aortic endothelial cells (MAECs) that oscillatory shear stress dramatically increased the production of endothelial cell superoxide $\cdot O^{2-}$ [5]. Hwang et al. (2003) supported this statement in BAECs claiming that oscillatory flow produces greater oxidative stress by $\cdot O^{2-}$ production, which enhances LDL oxidation and upregulates inflammatory markers [6]. Additionally, Ziegler et al. (1998) demonstrated in BAECs that oscillatory flow significantly contributed to endothelial dysfunction via significant increases in endothelin-1 and decreases in nitric oxide [7]. These adverse responses to an oscillatory pattern will eventually lead to cellular toxicity, smooth muscle cells proliferation and atherogenesis.

While in vitro studies have provided valuable insight into the consequences of retrograde and oscillatory shear stress, they can't be directly extrapolated to the in vivo human circulation. To address this issue, Thijssen et al. (2009) evaluated the effect of incremental levels of retrograde shear in the large arteries of young men [8]. These authors demonstrated that acutely exposing the brachial artery to retrograde shear significantly decreased endothelial-dependent flow mediated dilation (FMD). Additionally, they observed a linear relationship between FMD impairment in the brachial artery and the degree of retrograde shear. Similar findings have been reported in spinal cord patients. Totosy et al. (2015) concluded that 30 min of oscillatory shear

rate acutely impaired FMD% in the brachial artery and superficial femoral artery (SFA) in this population [9].

While the original work of Thijssen et al. (2009) suggested that prior exposure to retrograde shear decreases resting brachial artery endothelial function, it remains unknown whether microvascular function is also affected. The microcirculation or microvascular network refers to the compound of blood vessels where the exchange of gases (CO_2, O_2) and, nutrients and metabolic products are being perfused from the blood stream to the muscle tissues and vice versa. Recently, Near Infrared Spectroscopy (NIRS) has been used to monitor the redox state of hemoglobin and myoglobin within the muscle microvascular network [10] and has previously been used to evaluate microvascular reactivity in patient populations. Investigations of tissue oxygen saturation following a short period of vascular occlusion have revealed a decreased microvascular reactivity in patients with septic shock [11]. Kragelj et al. (2001) compared patients with peripheral vascular disease to healthy subjects via NIRS, measuring the oxygenation index (OI), which represents the net hemoglobin oxygenation status within the interrogated tissue [12]. Their NIRS measurements detected oxygenation changes in deeper tissues microvasculature and provided better dynamic signal than transcutaneous pulse oximetry ($TcpO_2$). Thus, NIRS was concluded to reflect closely the real response of the peripheral microvasculature to the occlusion stimulus.

Elucidation of the altered tissue perfusion characteristics, via NIRS measurements, in response to different hemodynamic shear rates, will therefore provide valuable insight into microcirculatory consequences of different magnitudes of

retrograde shear of microvascular function and changes in shear rate patterns. However, the impact of changes in retrograde shear stress to our knowledge has not been previously examined in humans at the microcirculatory level. Therefore, the main purpose of the present investigation was to determine the impact of acute changes in retrograde shear rate on microvascular function in healthy, young humans. It was hypothesized that exposure to acute increases in limb retrograde shear would decrease microvascular reactivity assessed via NIRS by decreased in the rate of resaturation, time-to-peak resaturation, and peak saturation. To test this hypothesis, microvascular reactivity was examined in the leg of healthy participants before and after a 30 min exposure to an increase in limb retrograde shear. The retrograde shear stimulus was achieved in one leg via inflation of a pneumatic cuff located below knee (75 mmHg), following the protocol used by Thijssen [8]. The contralateral leg served as a control.

Research aim

Determine the effect of acute changes in retrograde shear rate on microvascular function in healthy, young humans as determined via the post-occlusive NIRS response.

Hypotheses

(1) Alternative hypothesis: Exposure to incremental levels of retrograde shear rates will elicit a decrease in microvascular reactivity via NIRS by

i) decreases in the rate of resaturation,

ii) decreases time-to-peak resaturation and

iii) decreased peak saturation

(2) Null hypothesis: Exposure to incremental levels of retrograde shear rates will not result in a decrease in microvascular reactivity.

Significance of the study

Vascular function is a well-recognized predictor of cardiovascular diseases. Shear stress has the ability to induce changes in endothelial function, which can potentially lead to the presence of atherosclerosis. Results provided by flow-mediated dilation technique showed that anterograde shear stress improves macrovascular endothelial function. However, retrograde and oscillatory shear stress in both animal and human models induces pro-atherosclerotic mechanisms and macrovascular endothelial dysfunction. At the macrovascular level (e.g., brachial artery), the effect of different shear stress patterns on the endothelial function has been well studied, but no study to date has explored the effect of acute retrograde shear stress on microvascular level (i.e., arteriole and capillary). Therefore, the primary aim of the present study is to examine the impact of retrograde shear on microvascular function in vivo.

Assumptions

(1) Participants agreed and signed up inform consent forms.

(2) Participants followed the pretesting guidelines provided, such as caffeine and alcohol use or sports practice which influence the FMD outcomes.

(3) Responses of the recruited participants reflected the general population

(4) NIRS assumptions: Myoglobin did not influence the signal, the optodes were placed in the same position pre and post retrograde shear intervention, the light

did not cause any noise of the instrument and the part of the muscle studied represented the entire muscle.

Delimitations

(1) Participants were aged between 18 and 45 years old.

(2) Participants were recreationally active.

(3) Participants were selected from the Norman Campus at the University of Oklahoma.

(4) Participants were free of known cardiovascular disease

Limitations

(1) Protocol limited to rectus femoris muscle at microvascular level.

(2) Protocol limited to young, healthy participants.

Operational definitions

Shear stress: longitudinal pressure that the blood exerts against a blood vessel when flows through it.

Vascular endothelium: layer compound of endothelial cells that covers the internal part of a blood vessel and plays multiple physiological and pathological roles.

Flow-mediated dilation (FMD): shear-mediated dilation, technique used for evaluating vascular reactivity or the capacity of an artery to dilate in response to endothelial stimulation via increases in shear stress, therefore it evaluates vascular function.

Nitric oxide (NO): antioxidant, anti-inflammatory mediator and specially vasodilator,

vasoactive substance released by the endothelium because of an increase in shear stress leading to an increase in the blood vessel diameter.

Laminar flow: flow of blood across the vessel in the same direction, with the highest velocity found in the center of the vessel, happening in straight regions of arteries.

Turbulent flow: blood flow pattern characterized by not flowing linearly and smoothly, happening in the branches, bifurcations and curvatures of the arterial circulation.

Anterograde/antegrade shear: blood being directed from the heart to the periphery, associated with positive arterial adaptations and endothelium anti-atherogenic effects.

Retrograde shear: blood experiences a return towards the heart or backwards due to downstream resistance, believed to induce acute attenuation of endothelial function and to have pro-atherogenic effects.

NIRS (Near Infrared spectroscopy): technique based on infrared light to quantify the concentration of hemoglobin and myoglobin associated and free of O_2 in a specific portion of a tissue.

Tissue saturation index (TSI %): measurement that provides an average of the saturation of hemoglobin in all vascular compartments (arterioles, capillaries and venules) within the tissue. Similar annotations include tissue oxygenation index (TOI), tissue saturation (StO_2), and oxygenation index (OI).

Chapter 2: Literature review

Shear Stress and Flow-mediated dilation

Blood flow has been shown to be regulating factor in determining endothelial health and function, which in turn, is significantly related to cardiovascular function and pathophysiology, such as atherosclerosis[13].

When blood flows through a blood vessel, it exerts pressure parallel to the vessel wall and longitudinally in the direction of the blood flow. This longitudinal pressure is typically called shear stress. Shear stress is determined by several factors, including the velocity and pattern of the blood flow (Vd), the diameter of the blood vessel (D) and the viscosity of the blood (Figure 1). This shear stress directly influences the vascular endothelium, which plays multiple pathological and physiological roles, including the regulation of smooth muscle tone, control of thrombosis, inhibition of leukocyte and platelet cell adhesion, and promotion of intra-arterial permeability [13]. In addition, an acute increase in shear stress releases many vasoactive substances such as prostacyclins, endothelins, endothelial cell growth factors, interleukins, plasminogen inhibitors, and in particular nitric oxide (NO) [13].

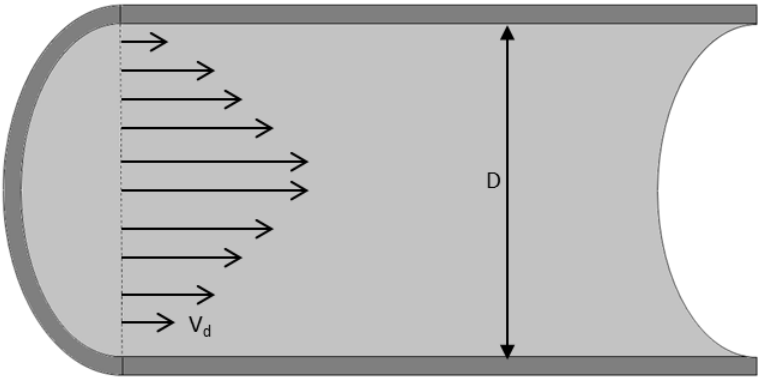


Figure 1 – Blood velocity profile in a ridged blood vessel

Traditionally macrovascular endothelial function is measured via flow-mediated dilation (FMD) since it evaluates the capacity of an artery to dilate in response to endothelial stimulation via increases in anterograde shear stress. The FMD protocol consists of inflation of a blood pressure cuff to a supra-systolic pressure for 5-min so that an ischemic stimulus appears and blood flow is interrupted [14] . After deflating the cuff, a reactive hyperemia or increase in blood flow to the limb is produced and the artery's diameter increases in response to the increases in blood flow and subsequent shear stress. This large increase in anterograde shear stress elicits endothelial derived release of NO, which elicits brachial artery dilation. Therefore, FMD is a largely endothelium- and nitric oxide (NO)-dependent stimulus [15] and it is measured using Doppler Ultrasound. Most studies assessing FMD use the brachial artery or the femoral artery.

Laminar vs. Turbulent Flow

In straight regions of arteries, the flow of blood across the vessel moves in the same direction, with the highest velocity found in the center of the vessel (Figure 1). This type of flow is known as laminar flow.

In regions of high antegrade laminar flow, endothelial cells have an anti-inflammatory phenotype characterized by alignment in the direction of flow, expression of anti-inflammatory genes, and low levels of oxidative stress, cell turnover and permeability; which make them protected from atherosclerosis[16] . These properties are potentially related to the capacity of high anterograde shear stress to induce adaptive dilation or structural remodeling of the artery wall through endothelium-mediated

mechanisms [17]. While the underlying mechanistic processes are not fully understood, some of them have been elucidated. One potential contributor is endothelial nitric oxide synthase (eNOS) which is one enzyme that is fundamental for the synthesis and release of NO, a vasodilator, antioxidant and anti-inflammatory mediator. Here is where the role of exercise takes place as a powerful weapon against atherosclerosis since it increases the blood velocity profile, causing a greater antegrade shear stress and therefore a greater NO production, with all its subsequent positive effects.

Unlike the flow pattern in long straight blood vessels, the branches, bifurcations and curvatures of the arterial circulation, cause the flow pattern to change such that blood does not flow linearly and smoothly (Figure 2). This type of flow pattern is called turbulent flow. Endothelial cells in regions of oscillatory flow have an activated, pro-inflammatory phenotype characterized by poor alignment, high turnover, oxidative stress and expression of inflammatory genes; make them highly susceptible to atherosclerosis [16].

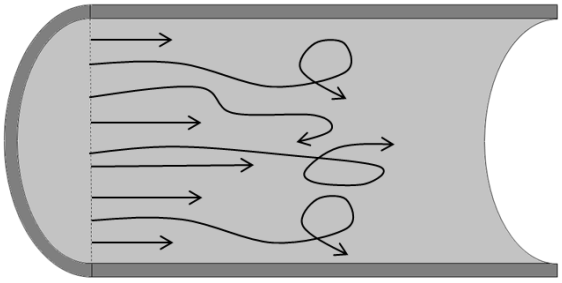


Figure 2 – Turbulent blood flow profile

Anterograde vs. Retrograde Flow/Shear Stress

The blood flow pattern within a cardiac cycle is characterized by a large anterograde shear during systole and a retrograde shear in early diastole, followed by a subsequent phase of anterograde flow in mid and late diastole (Figure 3)[18]. Anterograde shear refers to blood being directed from the heart to the periphery; whereas retrograde means that the blood experiences a return towards the heart or backwards, caused by downstream resistance.

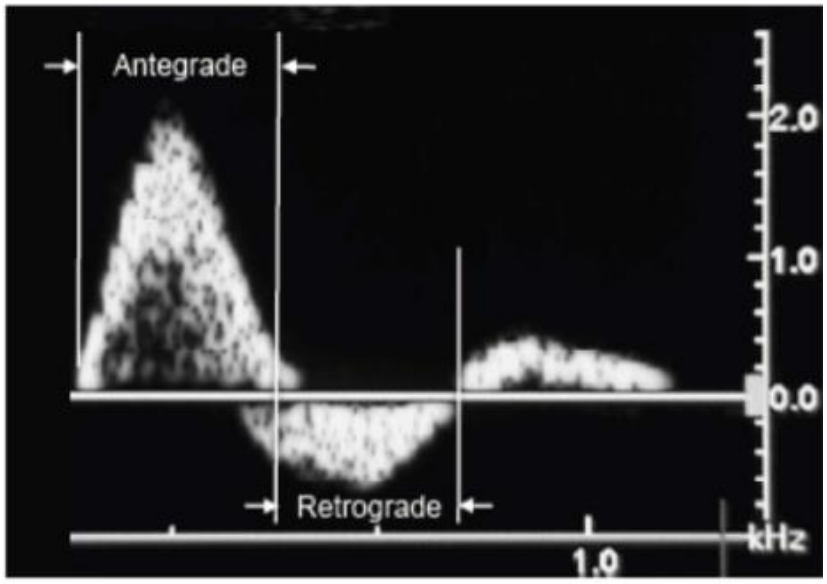


Figure 3 – Doppler waveform with antegrade and retrograde velocities identified.

Long term exposure (> a few minutes) to anterograde and retrograde shear might have different effects on endothelium and vascular function. Anterograde shear stress is believed to be beneficial due to its positive arterial adaptations and anti-atherogenic effects on the endothelium. On the contrary, retrograde shear stress is thought to induce acute attenuation of endothelial function and to have pro-atherogenic effects [14].

The extent to which incremental levels of retrograde shear effect endothelial function has been partially investigated. It has been shown that brachial artery FMD was impaired in a dose dependent manner in response to incremental levels of retrograde shear in young men (24 yr.). The highest level of retrograde shear, applied for 30min, resulted in the greatest the impairment in FMD [8] . These findings strongly suggest that an acute increase in retrograde shear in conduit arteries results in decreases in endothelial function in vivo, which is associated with the atherosclerotic process.

Another similar study, but conducted in older men (68 yr.), examined the impact of a step-wise increase in retrograde shear stress on brachial and femoral artery endothelial function. However, old men, as opposed to young men in the previous mentioned study, did not demonstrate changes in endothelial function in either artery. This means the presence of different adaptations in the vasculature of young and older humans exposed to similar shear stress stimuli may exist. Additionally, these findings may be related to an insufficient increase in retrograde shear required to affect FMD, to a depressed sensitivity of smooth muscle cells, to an age-related increased arterial stiffness, or to an a priori lower FMD; all of which have been observed in older adults [14].

Aging and vascular health is an important area of research since healthy aging has been associated with greater presence of retrograde and oscillatory shear in both the brachial and femoral arteries. Since the biological pathways that alter vascular tone are influenced by aging, Padilla et al (2011) examined the role that NO bioavailability plays in regulating shear rate patterns in young and old adults. In a group of young men (26 yr.) and older men (61 yr.) resting shear rate patterns were compared before and after

infusion of L-NMMA (which inhibits the synthesis of NO). They determined that NO synthase inhibition increased brachial artery retrograde shear and oscillatory shear in the younger participants, such that their level of retrograde/oscillatory shear was not significantly different than that observed in older participants. These findings suggested that NO bioavailability contributes to the presence of retrograde/oscillatory shear in large blood vessels. Therefore, it was concluded that aged related increases in retrograde/oscillatory shear occur in part to decreases in NO bioavailability [19].

Also the impact of retrograde shear rate on endothelial function may differ between different upper and lower limb arteries. Schreuder et al. (2014) examined the acute impact of graded increases in retrograde shear on endothelial function in brachial artery (resistant artery) and femoral artery (atherosclerotic prone). They concluded that an increase in retrograde shear is followed by a decrease in endothelial function similar in both arteries, in coherence with Thijssen et al. (2009). Also they reported a dose-dependent decrease in FMD, in both arteries. Therefore, the adverse responses to retrograde shear were similar in both arteries.

The role that exercise induces in beneficial vascular adaptations has been also studied but little is known about the underlying mechanistic link between the shear stress stimulus and the adaptive responses. It has been observed that different types of shear stress patterns are present during various types of exercise, which has led investigators to wonder if different patterns of shear rate are associated with different vascular adaptations. For example, Tinken et al. (2009) determined if different flow and shear stimuli had influence on different acute changes in vascular function via FMD in the brachial artery. Three different 30 min interventions associated with different shear

rate patterns were used: recumbent leg cycling, forearm heating and handgrip exercise. The contralateral limb was subjected to the same interventions simultaneously with a cuff inflated to 60 mmHg to attenuate shear levels within subjects. The main findings from this effort revealed a direct relationship between an increase in antegrade shear rate and an increase in FMD. Therefore, FMD was concluded to be modulated by different shear rate interventions, mainly because of differences in the magnitude of antegrade flow and shear (normally associated with exercise) which acutely improve brachial artery endothelial function.

To further understand the role episodic increases in anterograde shear rate has on vascular function, Tinken et al. (2010) examined indices of endothelium-dependent brachial artery function and remodeling following 8 weeks bilateral handgrip exercise. Throughout the experiment, one arm received a normal blood flow response (i.e., increased anterograde flow and shear) and one arm was cuffed to 60 mmHg (i.e., prevented the increase in anterograde shear). The 8 weeks program induced a time dependent change in FMD and in arterial remodeling in the arm exposed to the increased anterograde flow and shear, but this response was not found in the cuffed arm. Thus, the main conclusion was than an increase in antegrade shear is an important stimulus for enhancement of endothelial function.

An interesting study, carried by Iwamoto et al (2013), tried to clarify the effect of hypoxia on blood flow patterns in the inactive limb during constant-load dynamic exercise. Since during this kind of exercise, systolic function didn't seem to be different than in normoxia at the same relative workload, hypoxia may have little effect on antegrade blood flow. Nevertheless, hypoxia increases muscle sympathetic nerve

activity leading to an increase in retrograde blood flow. As it was hypothesized, it was determined that hypoxia had no effect on antegrade blood flow in brachial artery, but hypoxia augmented retrograde blood flow in the inactive limb during exercise, being its magnitude proportional to increase in hypoxia.

Totosy et al. (2015) examined shear rate pattern and FMD in brachial artery and superficial femoral artery (SFA) in these subjects versus able-bodied men. 30 min of 75 mmHg cuff inflation on the dominant forearm and dominant thigh was used to assess the effects of augmented retrograde shear rate through its respective mentioned arteries; non- cuffed limbs were considered as controls. This study demonstrated that a 30 min of altered shear rate, especially enhanced oscillatory shear rate, acutely impaired FMD% in both arteries in both SCI and control groups. However, they were unable to create an exclusively retrograde shear rate stimulus with cuff inflation therefore they couldn't conclude which shear rate parameter (antegrade or retrograde) was responsible for this impaired FMD%. Since retrograde shear rate was not different between groups, it wasn't the unique factor, so it may not contribute to impaired SFA FMD% in SCI. The authors also reported that the relationship between brief shear rate alterations and acute changes in FMD% was preserved in SCI, meaning that shear rate patterns may have implications for endothelial health regardless of neural vascular input. To sum up, in SCI, a tendency towards a higher retrograde shear rate response was observed, due to maybe disrupted sympathetic innervation and/or stiffer arterial walls after SCI.

Cellular effects of shear stress

In vivo studies provide information on the integrative effects of altered mechanical shear stress, but have their limitations. However, the utilization of endothelial cultured cells, in elastic tubes along with the application of mechanical stresses has provided detailed mechanistic insight into the effects of shear stress.

Ziegler et al (1998) exposed bovine aortic endothelial cells (BAECs) to either non-reversing pulsatile shear stress (i.e., primarily anterograde shear) or highly reversing shear stress (i.e., retrograde shear) to assess the effect of these different patterns on the expression of endothelin-1 (ET-1), endothelial constituve NOS (ecNOS mRNA) and ecNOS protein. ET-1 is a potent vasoconstrictor and is involved in the development of atherosclerosis. They showed that pulsatile shear stress can independently affect ET-1 mRNA expression in endothelial cells, but not ecNOS expression. In addition, unlike the unidirectional shear stress, which acted to enhance elongation and alignment in response to cyclic stretch, 24h exposure of BAECs to oscillatory stress did not produce those effects. These results lead led the authors to conclude that oscillatory flow and subsequent shear contributed to endothelial dysfunction because it increased ET-1 and decreases NO production, followed by cellular toxicity, smooth muscle cells proliferation and atherogenesis.

Uematsu et al (1995) in another article assessed BAEC, but this time versus human aortic endothelial cells (HAEC), to examine the characteristics of shear stress-induced ecNOS mRNA expression. They showed that two mechanisms could enhance ecNOS expression in the endothelium in response to shear stress: activation of protein kinase C (PKC) and the opening of K channels. The authors were also one of the first to

highlight the anti-atherogenic effects of endothelium-derived NO. They also highlight relationship between regions of high shear stress with anti-atherosclerosis outcomes, and low shear stress regions with higher atherosclerosis risk.

In 1999, Woodman et al studied the responses of endothelial Cu/Zn superoxide dismutase (SOD) in single coronary arterioles to shear stress. They determined that cultured endothelial cells exposed to shear stress had an upregulated SOD mRNA expression. SOD was also related to a reduction in the rate of NO degradation, which along with an increased ecNOS mRNA expression, may mediate NO-dependent vasodilation. The author's main findings were than an increase in ecNOS mRNA expression and SOD mRNA expression in coronary arterioles occurred following exposure to high flow/shear. Neither gene was induced by low flow/shear environment. Therefore, it was concluded that the increase in ecNOS mRNA and SOD mRNA expression could be stimulated in coronary arterioles by an increase in intraluminal flow, possible by an increase in shear rate, leading to improved NO- dependent vasodilation and increased blood flow.

In relation also with eNOS, Tuttle et al (2001) studied the relationship between rate and magnitude of rapid luminal expansion (produced in small resistance arteries that form collateral pathways after abrupt arterial occlusion) and the degree of initial shear increase. Rapid luminal expansion and specific remodeling events within the arterial wall layers (such as endothelial cell proliferation and smooth muscle cell hypertrophy) occurred when shear forces were at their highest, but as it returned to normal levels, rate decreased and endothelial cell hyperplasia was observed. Their conclusion was that the rate of luminal expansion, specific remodeling events within the

wall layers, and the degree of endothelial gene expression were influenced by the level of blood flow or wall shear alteration.

Assessment of Macro- and Microvascular Function

Flow-Mediated Dilation

FMD is used as a non-invasive technique to measure endothelial dysfunction and has also been proposed to represent a functional bioassay for endothelium-derived NO bioavailability in humans.

During an FMD test, a cuff is inflated producing suprasystolic occlusion for a period of time, which creates a region of ischemic tissue distal to the point of occlusion. The metabolic byproducts of cellular respiration, in the absence of circulating blood, promote an increase in vascular conductance that allows a robust hyperemia when the cuff is released. This hyperemia increases laminar shear forces parallel to the long axis of the vessel, which is transduced via luminal mechanoreceptors to the endothelial cell and will at the end signal an increase of eNOS activity and NO production. NO will induce relaxation of the smooth muscle and subsequent vasodilation [17].

The longer the duration of the occlusion and the more proximal the cuff is placed, the greater is the degree of ischemia and the greater the hyperemia. Nevertheless, despite the apparent link between FMD and NO bioavailability, there is still some debate regarding whether vasodilation mediated by the endothelium is predominantly a consequence of NO.

It is also important to consider that there are other vasodilators in response to shear stress aside from NO. For example, prostaglandin and endothelial-derived

hyperpolarizing factor (EDHF) responses have also been observed mechanisms underlying the FMD response in humans. Indeed, EDHF is believed to compensate when NO production is compromised. Pyke et al. (2009) blocked NO with L- NMMA, and it was found to be not obligatory for FMD in response to the reactive hyperemia following 5 or 10 min of occlusion in young healthy males.

FMD is assumed to reflect NO bioavailability, but if its contribution to the response is not mandatory and can be compensated for, then the degree to which FMD reflects NO bioactivity is highly variable. So the use of FMD as a specific bioassay for NO may need to be reconsidered. Given that there is clearly a relationship between vascular health and FMD, this suggests that NO bioavailability is not the only mechanism responsible.

Methodologically there is no consensus as to the exact placement of the occlusion cuff relative to the site of measurement. Green et al. (2011) found that FMD is highly NO dependent when the brachial artery lies upstream from the occluding cuff (distal cuff) but less NO dependent if the imaged artery lies within the ischemic territory during occlusion (proximal placement). However, proximal cuff placement is more predictive of cardiovascular events, challenging the idea again that FMD owes its prognostic capacity to its assessment of NO bioavailability [20]. This paper ended up concluding that any direct measure of endothelial function may provide independent prognostic information in humans, which compliments that available from traditional risk factor assessment.

Supporting this idea, Wray et al. (2013) stated that FMD testing is a valid assessment of disease risk and progression, although the role of NO in the FMD

response is not clear yet. Actually, these authors suggest that the FMD response should be viewed as endothelium-dependent vasodilation, but not necessarily NO-dependent vasodilation. Also, they mentioned that it seems reasonable to speculate that prostaglandins and EDHF may play an important compensatory role in the FMD response, particularly in the absence of NO. However, in contrary to the previous mentioned study, they refer to the present guidelines for FMD testing in which the occlusion cuff is placed distal to the measurement site, since proximal cuff placement exposes the vessel to an ischemic environment that may provoke vasodilation, which is not endothelium dependent.

Additional methodological procedures should also be considered. 1) To inflate and deflate the occlusion cuff a rapid (~0.3 second) cuff inflator is recommended. Along with this, the bump caused by the cuff procedure should be taking into consideration and it can be avoided by supporting the limb in a way that there is adequate space under it for the cuff to inflate and deflate. 2) Regarding to the duration of occlusion, for the sake of consistency and subject comfort, it is standardized procedure that the cuff is inflated ≥25 to 50 mmHg above systolic arterial pressure for 5 minutes. However, the duration of cuff occlusion is debatable since some evidence supports that ≥5 minute occlusion may include vasodilators not associated with NO. 3) The increase in arterial diameter, as a consequence of the reactive hyperemia, is compared with the baseline diameter and expressed as a percentage of this baseline diameter (% FMD). However, since the initial baseline diameter could introduce mathematical bias into the FMD assessment, with smaller vessels appearing more reactive researchers should document baseline diameters, absolute change in diameter,

and shear rate (AUC). 4) It has been suggested that FMD should be normalized by dividing the percentage of FMD by shear rate since the shear stress/rate is the stimuli driving the FMD response. Many researchers have embraced this mathematical adjustment, but it accuracy in describing the physiology remains uncertain. It is recommended that FMD normalized for AUC be calculated and reported, but that the raw shear (AUC up to peak vasodilation) and FMD data also be readily available to allow for further analyses or interpretations. 5) When it comes to FMD measurement, the angle of insonation, which is the angle between the transducer and the studied vessel, is of key relevance. This angle determines blood velocity and subsequent calculation of blood flow, so it has been standardized as 70 degrees. Differentiating between angle of insonation and angle correction is also important. Angle correction specifies the true Doppler angle by placing the cursor parallel to the direction of blood flow [21].6) Arterial diameter can vary quite considerably across a single cardiac cycle, that's the reason why most ultrasounds Doppler have an integrated electrocardiogram (ECG) so it facilitates the assessment of diameter according to the cardiac cycle. But if it is not available on the ultrasound system itself, an external ECG can be used. 7) After resting state, baseline measurements should be performed. For calculation of baseline arterial diameter, it is recommended that ≥10 cardiac cycles are used and because FMD is based on change in diameter, there is a need for consistency from baseline until maximal dilation. Besides, it is recommended that baseline blood velocity is averaged over at least a 10 to 20 second period, and to optimize the insonation angle, placement and size of the sample volume (gate width) are of critical importance. Therefore, it is recommended that the sample volume be as wide as possible but without encompassing

the vessel walls and allowing for a slight margin for error in case of movement. 8) Post-cuff measurements are recommended to be initiated ≥10 seconds before cuff release. Due to individual differences, it is recommended that the true peak diameter be determined on an individual basis and the time to peak vasodilation be reported. Besides, to truly assess the shear that reactive hyperemia induces and subsequent vasodilation, it is recommended that blood velocity and diameter measurements be performed for ≥2 minutes after cuff release. 9) The use of edge detection software for offline analysis is recommended for the measurement of baseline and post-cuff release diameters because provides a more robust and sensitive assessment of FMD and removes any subjective error component from the data analysis [17].

The physical state of the participant is also very important to consider prior to testing. The following factors should be considered.

- Vitamin supplementation should not be ingested 72 hours before FMD assessment because pro-oxidant and antioxidant balance plays a clear role in vascular endothelial function. In the same line, but more difficult to control, a diet high in antioxidants may also influence the results of an FMD.
- Subjects should refrain from taking all medications for ≥4 half-lives of the drug before FMD assessment. In particular the investigator should be aware of those drugs with effects in the cardiovascular system, such as beta-blockers. In case of mandatory intake of the medication, everything should be recognized and documented.

- Tobacco has been documented to attenuate endothelial function, so it is recommended that subjects refrain from both smoking and smoke exposure for ≥12 hours before FMD measurements.
- Another substance that attenuates FMD, because it inhibits a step in the NO-mediated process that results in vasodilation, is caffeine. Therefore, no ingestion of caffeine should be allowed ≥12 hours before FMD testing.
- When studying women FMD, measurements should be performed days 1 to 7 of the menstrual cycle to minimize the impact to these hormonal changes since low levels of both estrogen and progesterone in women are found in that period.
- Subjects abstain from exercise for ≤12 hours before an FMD measurement since a single bout of exercise has been documented to improve FMD.
- FMD assessments should be performed under fasting conditions since different types of diets have different effects on endothelial function. For example, the consumption of a single high-fat and high-carbohydrate meal has been shown to attenuate FMD.
- Since FMD measurements are done comparing with baseline values, true baseline should be accurately assessed. Thus, the subject should remain in the position in which the study will be performed for ≥20 minutes in a quiet, climate controlled room (22°C to 24°C) before the actual measurement. Moreover, it is recommended a separate familiarization visit of the procedures to limit the stress that sympathetic activity can induce on the day of actual measurement.
- -Regarding repeated measurements, it has been documented that multiple FMD tests can be validly performed if ≥30 minutes separate each measurement.

Important to consider is that FMD measurements exhibit diurnal variation, and, thus, comparisons between and within subjects should be performed as consistently as possible with regard to the time of day.

- After resting state, baseline measurements should be performed. For calculation of baseline arterial diameter, it is recommended that ≥10 cardiac cycles are used and because FMD is based on change in diameter, there is a need for consistency from baseline until maximal dilation. Besides, it is recommended that baseline blood velocity is averaged over at least a 10 to 20 second period, and to optimize the insonation angle, placement and size of the sample volume (gate width) are of critical importance. Therefore, it is recommended that the sample volume be as wide as possible but without encompassing the vessel walls and allowing for a slight margin for error in case of movement.

Microvascular function via Near-infrared Spectroscopy (NIRS)

Dysfunction within the vascular system can occur in either the macrocirculatory or the microcirculatory level, with a reduce oxygen delivery being a potential cause for functional and structural disturbances of organs and tissues [12] . To date several methods have been developed for the non-invasive evaluation of the peripheral microcirculation. One new non-invasive, easily applicable technique currently under use for the evaluation of microvascular tissue oxygenation and hemodynamics is Near Infrared Spectroscopy (NIRS). This technique uses light in the infrared region and has the capacity to penetrate skin and bone and reach deeper structures (i.e., muscle tissue). In muscle tissue, near infrared light is absorbed and scattered by chromophore

molecules. The most important chromophores are hemoglobin (Hb), myoglobin (Mb) and water. The wavelength range used is 690 nm - 830 nm since wavelengths above 900 nm are absorbed by water and wavelengths below 690 nm don't penetrate the tissue. Water is considered to have a constant concentration, so it doesn't interfere in the measurement. The absorption characteristics of deoxyhemoglobin (HHb) and oxyhemoglobin (HbO_2) are different, so when light of different wavelengths is emitted, it is absorbed to a different degree depending on the relative concentrations of those [22]. In other words, the fact that O_2 is present in these molecules alters their absorption spectra so the determination of Hb and Mb containing O_2 is possible. It is important to mention that NIRS can differentiate from oxygenated and deoxygenated chromophores but not between Hb and Mb because both have very similar absorption spectra [23]. Despite of this disadvantage, Mb O_2 desaturates almost totally during exercise in either normoxic breathing room air (21% PO_2) and hypoxia condition (12% PO_2) , and so any changes during exercise is attributed primarily to changes in Hb oxygenation and deoxygenation [24].

NIRS technology can be used to quantify the concentrations of: Total microvascular hemoglobin + myoglobin concentration ([Hb] total), 2) oxygenated microvascular hemoglobin + myoglobin concentration ([HbO_2]), 3) deoxygenated microvascular hemoglobin + myoglobin concentration ([HHb]) and 4) Tissue Saturation Index (TSI%) = [HbO_2] / ([HbO_2] + [HHb]) in the microvascular compartments (arterioles, capillaries and venules) within the interrogated tissue.

Vascular Occlusion Test and microvascular reactivity

The vascular occlusion test is a commonly test that allows for non-invasive evaluation of microvascular function. Similar to the previously described FMD procedure, a rapid inflating cuff is placed on the upper arm and inflated for 5 minutes. When the cuff is released the reactive hyperemic response can be evaluated via a NIRS system and used to evaluate microvascular reactivity.

During the procedure, the NIRS probe should be placed ensuring adequate sealing to avoid the ambient light from interfering with the values. Use of adhesive tape is acceptable, but it cannot be too tight because it can apply pressure on the microvasculature of the underlying tissue.

TSI% baseline values at rest are recorded first and then, the cuff should be rapidly inflated to a pressure above the systolic blood pressure inducing arterial and venous occlusion. During the vascular occlusion, it is important to state that pain could affect the measurement producing vasoconstriction, so no pain should be reached. Upon cuff release all measurements should be continued until the return of TSI% to baseline values.

After deflating the cuff, the rate of TSI% increases, which reflects the resaturation rate of hemoglobin, which depends on reperfusion rate, which in turn depends on microvascular function. It is important to denote here that NIRS measures resaturation and not reperfusion. In total, the degree of reactive hyperemia assessed via NIRS depends on the ability of the microcirculation to respond adequately to the ischemia.

Assessment of microvascular reactivity via the vascular occlusion technique and NIRS technology can be used as a prognostic index in critically ill patients as well as a monitoring tool [22]. Kragelj et al. (2001) compared patients with peripheral vascular disease against healthy subjects. They determined that the recovery times after arterial occlusion for HbO_2, HHb, and TSI% were significantly longer in the PVD patients compared to controls. Additionally, these parameters were signifincaly correlated with measurements of ankle-brachial index, a clinical measure in PVD patients.

De Blasi et al. (2005) investigated microvascular function, regulation, oxygenation, and cellular metabolism in the human brachioradial muscle during subacute septic shock. These authors report that reperfusion time following the vascular occlusion period lengthens with septic shock. These findings suggested a maldistribution, limitation of microvascular blood flow or both irregularities during septic shock.

Chapter 3: Methods

Ethical Approval

The study protocol was approved by Institutional Review Board for Research Involving Human Subjects at the University of Oklahoma, and conformed to the Declaration of Helsinki. Each potential participant was screened to determined potential eligibility by the research team. If the individual was eligible they were asked to consider joining the study. Individuals who met the initial eligibility criteria and who agreed to participate in the study signed informed consent, appropriate HPPA forms, and complete Medical History Form.

Participants

This study utilized a randomized balanced double-blind placebo-controlled crossover study design. A total of 17 participants (men (10) and women (7), age 22.7 ± 2.91 yr, height 175.11 ± 10.46 cm, body mass 74.02 ± 13.52 kg) volunteered for the study. Females were tested on days 1 to 7 of the menstrual cycle due to known effects of the menstrual cycle on peripheral cardiovascular function. Participants were recruited from the campus of the University of Oklahoma (Norman, Oklahoma) via emails, flyers, and newspaper ads placed within the surrounding communities.

Inclusion Factors

(1) Men and women

(2) 18-45 years old

(3) Sedentary-to-recreationally active (i.e., less than 10 hours of vigorous physical activity per week)

Exclusion Factors

(1) Known atherosclerosis cardiovascular disease (ASCVD), defined by history of acute coronary syndromes, myocardial infarction, stable or unstable angina, coronary or other arterial revascularization, stroke, TIA, or peripheral artery disease.
(2) Known diabetes
(3) Known hypertension (>140 mmHg resting systolic blood pressure)
(4) Current smokers or within the last 6 months
(5) Current and chronic use of anti-inflammatory drugs (e.g., NSAIDS).

Experimental protocol

All experimental testing occurred in the principle investigator's dedicated laboratory located in the Houston Huffman Center on the University of Oklahoma-Norman campus. All testing was conducted in a thermoneutral setting (21-23°C). Subjects arrived at the laboratory after a 6 h fast and having refrained from strenuous exercise, alcohol, and caffeine for 12 hours.

Microvascular function was assessed in each participant before and immediately after a 30 min shear rate treatment (Figure 4).

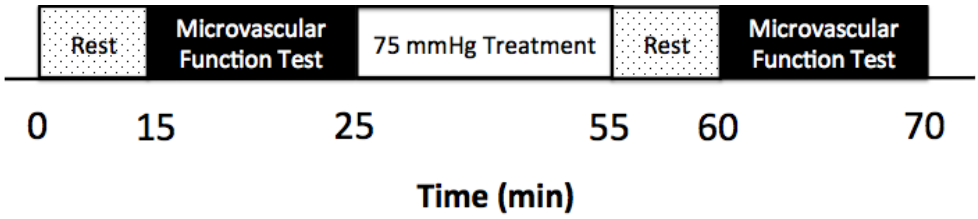


Figure 4 - Protocol

Retrograde shear treatment

Retrograde shear treatment was achieved in the randomly selected leg (Treatment leg) via a blood pressure cuff placed below the knee inflated to the target pressure of 75 mmHg in a similar method as previously described by Thijssen et al. for a 30 min period [8]. The contralateral leg served as the control/non-treatment leg (Control leg).

Superficial femoral artery diameter as well as blood velocity measures were obtained at the beginning, at the middle and ending of the shear rate treatment in both legs via two dimensional and Doppler Ultrasound with a linear array transducer operating in duplex mode and an insonation angle of <60 (Logic S8, GE Medical Systems, Milwaukee, WI). All measurements of arterial diameters and velocities were performed over a continuous 10 cardiac cycles. Mean (i.e., across the entire cardiac cycle), anterograde, and retrograde shear rates were calculated using mean, anterograde and retrograde blood velocities, respectively (Figure 6). The oscillatory shear index (OSI) was used to characterize the magnitude of the shear rate oscillations throughout a cardiac cycle. The OSI is calculated as: OSI = |retrograde shear| / (|anterograde shear| + |retrograde shear|) such that OSI values of zero correspond to unidirectional shear rate

while values of 0.5 are indicative of oscillations with a mean shear rate of zero ([9],[25]).

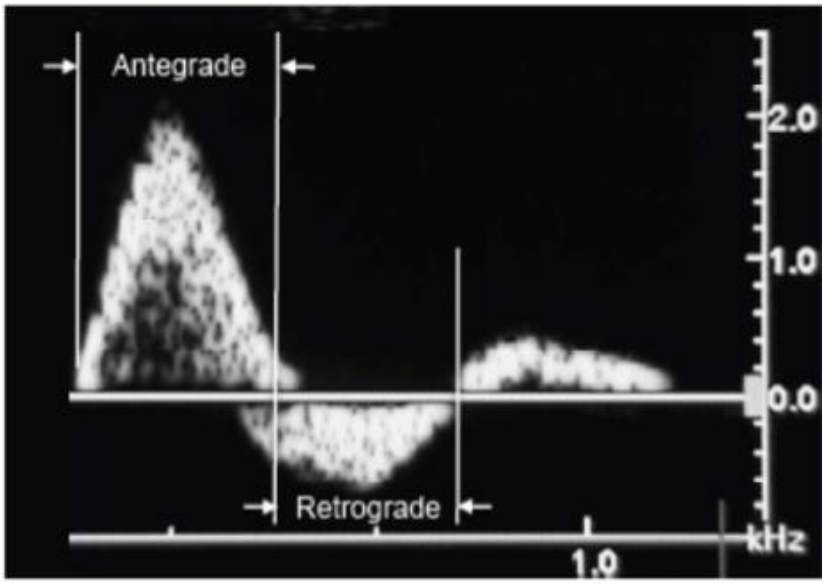


Figure 5 - Doppler waveform. Note that anterograde blood velocities occur above baseline and retrograde occurs below baseline.

Experimental Measurements

Anthropometric Measurements

Participant's height was measured on a standard digital scale (ProDoc Dectecto stadiometer). For this purpose, the participant stood up right against the wall with no shoes or hat, making sure his or her heels were touching the wall. Then, the flexible arm from the scale was placed above the participant's head to record real height. Participant's weight was measured on a digital stadiometer (HealthOMeter 349KLX).

Microvascular function

Before and after the shear rate intervention microvascular function of the m. *rectus femoris* of the quadriceps muscle in the Treatment and Control legs were simultaneously determined using a state-of-the-art, rapid sampling frequency-domain multi-distance near infrared spectrometer (NIRS; OptiplexTS; ISS, Champaign, IL). In the present protocol, the NIRS probe was placed longitudinally on the belly of the muscle and correct placement was confirmed by obtaining an ultrasound image of the tissue below the probe. The data were sampled at 50 Hz and averaged into 1 sec bins off-line. No movement of the probe occurred during the procedures. Throughout the procedure, both Treatment and Control legs were rested straight at heart level and placed on a foam pad or towel.

Pre-intervention, each participant rested in the supine position for 15 min to allow for measurement of resting heart rate and mean arterial blood pressure. Resting heart rate and mean arterial blood pressure were measured in the supine position from the average of three recordings (Omron BP785N, Hoofddorp, Netherlands). Following the resting period a blood pressure cuff attached to a rapid inflation/deflation pneumatic system (D.E. Hokanson, Bellevue, Wash) was positioned on the upper leg at the level of the femoral artery. Baseline NIRS measurements were then made for 5 min, the pneumatic cuff was then inflated to >250 mmHg for 5 min. The microvascular measurements were made during this occlusion period and for an additional 5 min post-occlusion to ensure the entire response was recorded.

The microvascular post-occlusive response was calculated as depicted in Figure 5. Resaturation rate was quantified as the slope of the TSI%, [Hb]total, [HbO_2], and

[HHb] response following cuff release. The absolute peak and time to peak response for TSI%, [Hb]total, [HbO_2], and [HHb] were calculated from the highest 5 sec mean values following cuff releases.

Briefly the NIRS Probe consisted of eight light emitting diodes operating at two wavelengths (690 and 830 nm) and a single detector fiber bundle (source detector separation of 2.0-3.5 cm). This system provides absolute values of microvascular tissue hemoglobin + myoglobin saturation (TSI%), total concentration ([Hb total), and individual oxygenated ([HbO_2]) and deoxygenated ([HHb]) concentrations, all of which are used in combination for the evaluation of microvascular tissue hemodynamics.

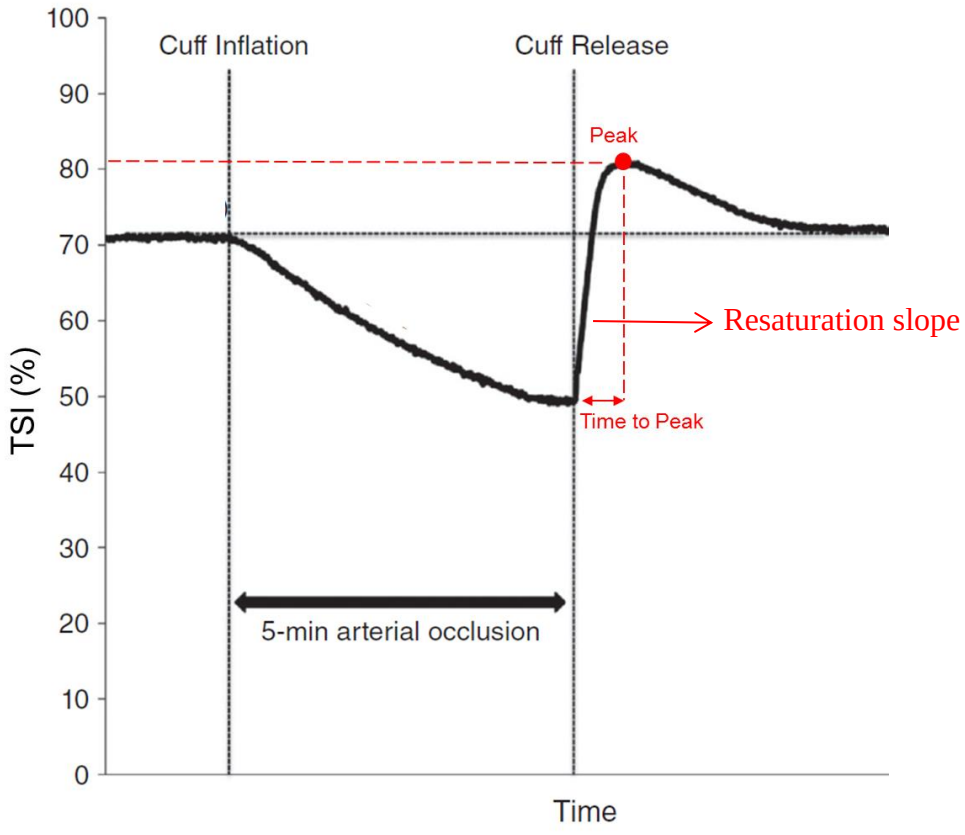


Figure 6 – Post-occlusive NIRS response for tissue saturation index (TSI%).

Statistics

The sample size was based on power calculations and reported samples sizes of similar studies investigating the microvascular function assessed via NIRS signal responses [12]. Our sample size utilized an alpha error rate of 0.05, a beta error rate of 0.20, which corresponds to a power of 80%, and an estimated effect size of 0.5 at least 15 to 25 subjects are needed.

A paired *t* test was used to compare shear rate responses between Pre-Intervention and during Intervention. A two-way repeated-measures ANOVA was used to compare Pre-Intervention and Post-Intervention NIRS responses between Control and Treatment legs. Post-hoc tests with a least-squares difference test were performed when the 2-way ANOVA reported a significant main or interaction effect. Significance was declared when $p<0.05$.

Chapter 4: Results

Participant Characteristics

Table 1 describes the subjects' characteristics. 17 subjects in total (10 men, 7 women), age 22.7±2.91 yr, height 175.11±10.46 cm and body mass 74.02±13.52 kg took part in the study. Systolic and diastolic blood pressures (n=16) at rest were 113.12 ± 11.56 mmHg and 68.56 ± 8.33 mmHg, while systolic and diastolic blood pressures after the retrograde shear intervention (n=16) were 114.66 ± 12.73 mmHg and 72 ± 8.22 mmHg, respectively. Heart rate (n=16) at rest was 66.18 ± 10.09 bpm and 66.18 ± 10.09 bpm following the shear rate intervention.

Table 1. Subjects characteristics

Variable	Value
n	17
Sex (men/women)	10/7
Age (years)	22.7 ± 2.91
Height (cm)	175.11 ± 10.46
Weight (cm)	74.02 ± 13.52
Body mass index (kg m^{-2})	24.03 ± 3.28
Systolic blood pressure (mmHg) at rest (n=16)	113.12 ± 11.56
After inducing retrograde shear	114.66 ± 12.73
Diastolic blood pressure (mmHg) at rest (n=16)	68.56 ± 8.33
After inducing retrograde shear	72 ± 8.22
Heart rate (bpm) at rest (n=16)	66.18 ± 10.09
After inducing retrograde shear	62.33 ± 8.71

Values are mean ± SD

Shear Rate Patterns in the NonCuffed (Control) and Cuffed (Treatment) Legs

Table 2 describes the results of changes in cardiac cycle shear, anterograde shear, retrograde shear, and oscillatory shear index in the control and treatment legs. Cuff inflation in the treatment leg elicited significant changes in the shear rate pattern (Figure 7). Mean shear rate across the entire cardiac cycle in the treatment leg was significantly decreased during the intervention ($36.46 \pm 16.33\ s^{-1}$) compared to Pre-Intervention ($62.36 \pm 26.05\ s^{-1}$). However, mean shear rate rate across the entire cardiac cycle in the control leg was $61.14 \pm 38.00\ s^{-1}$ pre-intervention and $69.77 \pm 34.77\ s^{-1}$ during the intervention without a significant difference ($P = 0.376$) .

Regarding antegrade shear rate, there was no difference between the Pre-Intervention and Intervention values in neither control leg ($P = 0.069$) nor in the treatment leg ($P = 0.410$). The control leg values yielded a $378.44 \pm 82.9\ s^{-1}$ value in the Pre-Intervention and a $353.65 \pm 65.82\ s^{-1}$ during the Intervention. The treatment leg values were $386.35 \pm 90.2\ s^{-1}$ pre-Intervention and $403.29 \pm 110.08\ s^{-1}$ during the Intervention.

Retrograde shear rate was significantly increased during the Intervention compared to Pre-Intervention in the Treatment leg ($P < 0.001$) but not within the Control leg ($P = 0.365$). In the control leg Pre-Intervention retrograde shear was $74.82 \pm 58.55\ s^{-1}$ and during the Intervention was $66.62 \pm 45.69\ s^{-1}$. In the Treatment leg Pre-Intervention value for retrograde shear was $71.18 \pm 46.78\ s^{-1}$ and during the Intervention it was $156.54 \pm 63.48\ s^{-1}$.

The Oscillatory Shear Index (OSI) was significantly different in both the Control and Treatment legs ($P < 0.001$). In the Control leg Pre-Intervention and

Intervention values were 0.15 ± 0.87 and 0.04 ± 0.54, respectively, which equates to a 73% decrease. Conversely, in the treatment leg Pre-Intervention and Intervention values were 0.15 ± 0.75 and 0.43 ± 0.07, respectively, equating to a 186% increase. The control oscillatory shear index is considered significantly different because of a lower influence of the sympathetic nervous system making muscle tone and blood vessels relaxed, explaining the decrease in the retrograde shear. Also it was influenced by the math calculation, since a decrease in both antegrade and retrograde components would make a significant difference in the end result (OSI).

Table 2. Vascular shear rate during experiment

	Pre-Intervention	Intervention	p-value
Cardiac Cycle shear rate (s^{-1})			
Control leg	61.14 ± 38.00	69.77 ± 34,77	0.376
Treatment leg	62.36 ± 26.05	36.46 ± 16,33	<0.001
Anterograde shear rate (s^{-1})			
Control leg	378.44 ± 82.9	353.65 ± 65,82	0.069
Treatment leg	386.35± 90.2	403.29 ± 110,08	0.410
Retrograde shear rate (s^{-1})			
Control leg	74.82 ± 58.55	66.62 ± 45,69	0.365
Treatment leg	71.18 ± 46.78	156.54 ± 63,48	<0.001
Oscillatory Shear Index			
Control leg	0.15 ± 0.87	0.04 ± 0.54	<0.001
Treatment leg	0.15 ± 0.75	0.43 ± 0.07	<0.001

Values are means ± SD

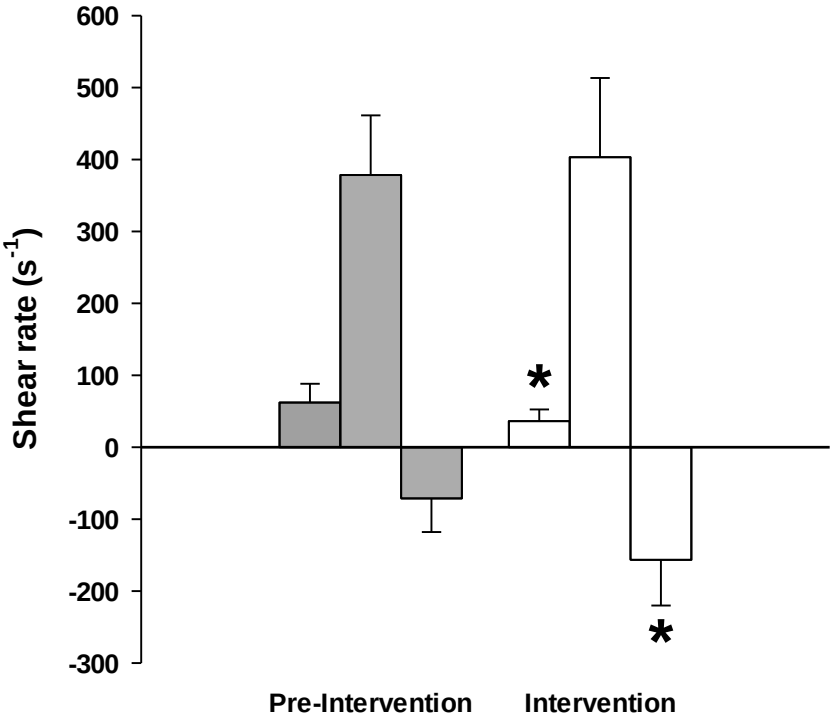


Figure 7 – Mean shear rate and shear rate pattern pre-intervention (solid bar) and during the intervention (open bar) in the treatment leg. (+ve, antegrade shear rate; -ve, retrograde shear rate) (mean ± SD)

Microvascular Function in the NonCuffed (Control) and Cuffed (Treatment) Legs

Baseline NIRS Results

Table 3 describes the results of the average NIRS variable estimates. Baseline TSI% was statistically increased Post-Intervention in the Treatment Leg compared to Pre-Intervention ($P = 0.024$). Baseline [Hb]total ($P<0.001$) and baseline [HbO2] ($P<0.001$) were also statically increased in the Treatment leg Post-Intervention compared to Pre-Intervention. Baseline TSI% ($P=0.23$) was unchanged Post-Intervention in the Treatment leg. Baseline [Hb]total ($P<0.001$) and baseline [HbO2]

(P<0.001) were also statically increased in the control leg Post-Intervention compared to Pre-Intervention.

Table 3. Average NIRS parameter estimates

Parameter	Control leg		Treatment leg	
	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention
BSLN TSI% (%)	66.14 ± 15.3	69.5 ± 11.5	66.93 ± 14.56	73.8 ± 9.6[α]
BSLN [Hb]total (µM)	39.05 ± 32.49	42.5 ± 33.8[α]	34.44 ± 24.52	37.7 ± 25.8[α]
BSLN [HbO2] (µM)	29.35 ± 29.77	32.2 ± 31.2 [α]	24.07 ± 19.48	28.6 ± 21.4 [α]
BSLN [HHb] (µM)	9.15 ± 6.40	10.23 ± 6.3	13.59 ± 16.62	9.14 ± 6.5
Slope TSI% (% s-1)	0.00079 ± 0.001	0.0005 ± .0006	0.0004 ± 0.0004	0.0006 ± 0.0007
Slope [Hb]total (µM s-1)	0.0002 ± 0.0003	0.0003 ± .0004	0.0002 ± 0.0004	0.0004 ± .0004 [α]
Slope [HbO2] (µM s-1)	0.0003 ± 0.0003	0.0003 ± .0004	0.0002 ± 0.0004	0.0004 ± .0005 [α]
Slope [HHb] (µM s-1)	-0.00005 ± -0.00008	-0.00006 ± .00007	-0.00003 ± -0.001	-0.00005 ± .0001
Peak TSI% (%)	75.58 ± 11.05	76.8 ± 11.9	74.24 ± 13.05	79.9 ± 10.2 [α]
Peak [Hb]total (µM)	46.87 ± 37.71	51.5 ± 43.3 [α]	40.28 ± 27.08	44.7 ± 29.7 [α]
Peak [HbO2] (µM)	38.16 ± 36.56	43.1 ± 43.6 [α]	30.23 ± 23.19	36.4 ± 26.6 [α]
TTP TSI% (s)	84.54 ± 31.39	72.23 ± 25.0	89.16 ± 44.19	82.15 ± 39.9
TTP [Hb]total (s)	72.85 ± 13.63	61.8 ± 34.1	77.40 ± 39.94	66.6 ± 32.5
TTP [HbO2] (s)	86.28 ± 32.98	67.0 ± 27.5	86.28 ± 42.13	77.9 ± 40.4
TTP [HHb] (s)	5.69 ± 10.13	4.7 ± 10.5	12.72 ± 25.81	14.53± 26.4

Values are means ± SD;Values are means ± SD; BSL, baseline; TSI%, hemoglobin + myoglobin saturation; $[Hb]_{total}$, total hemoglobin + myoglobin; $[HbO_2]$, oxygenated tissue hemoglobin + myoglobin; [HHb], deoxygenated hemoglobin + myoglobin; TTP, time-to-peak.

[α] Data are significant from Pre-Intervention at P<0.05

[β] Data are significant from Control Leg Post-Intervention at P<0.05

NIRS Resaturation Results

NIRS Resaturation variables are also provided in Table 3. Post-Intervention TSI% resaturation values weren't significantly different to Pre-Intervention in neither the Control nor the Treatment leg(P=0.872). Post-Intervention and Pre-Intervention [Hb] total resaturation didn't show any significant change in the Control (P=0.25) leg but the Post-Intervention value did increase in the Treatment leg (P=0.003). The same scenario, in which an increase in Post-Intervention versus Pre-Intervention was present in the treatment (P<0.001) but not in the control leg (P=0.15), applies for [HbO_2] resaturation. No significant change in [HHb] resaturation was found in any of the tested legs (P=0.08).

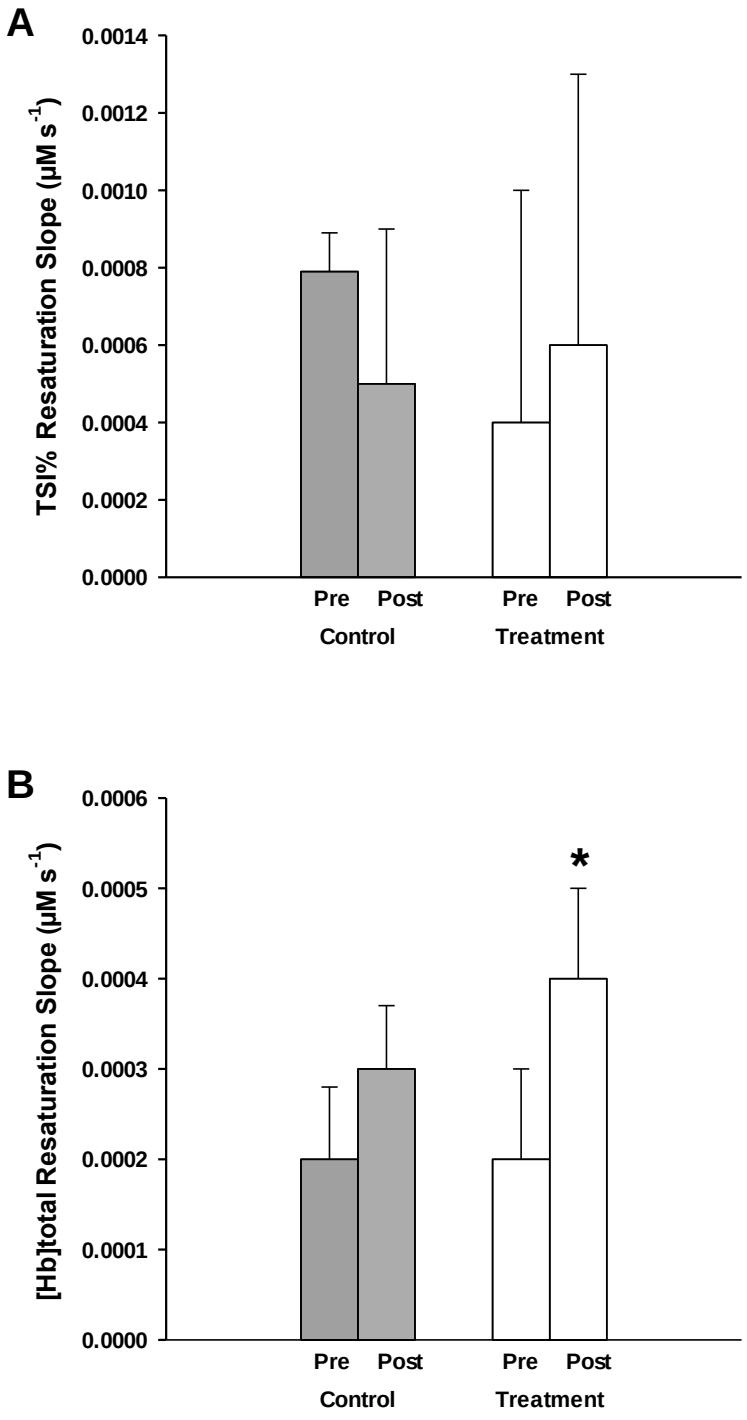


Figure 8 – (A) TSI% resaturate slope. (B) [Hb]total resaturation slope. * Significant versus pre. P < 0.05. (mean ± SD)

Peak NIRS Results

Post-Intervention peak TSI% was found to be increased relative to Pre-Intervention in the treatment leg ($P<0.001$), but such change does not occur in the control leg ($P=0.34$). Post-Intervention peak [Hb] total values were different compared to Pre-Intervention values in both control ($P=0.002$) and treatment legs ($P=0.003$), being the Post-Intervention values higher. In the same way, peak Post intervention [HbO2] values increased compared to Pre-Intervention in both legs ($P<0.01$).

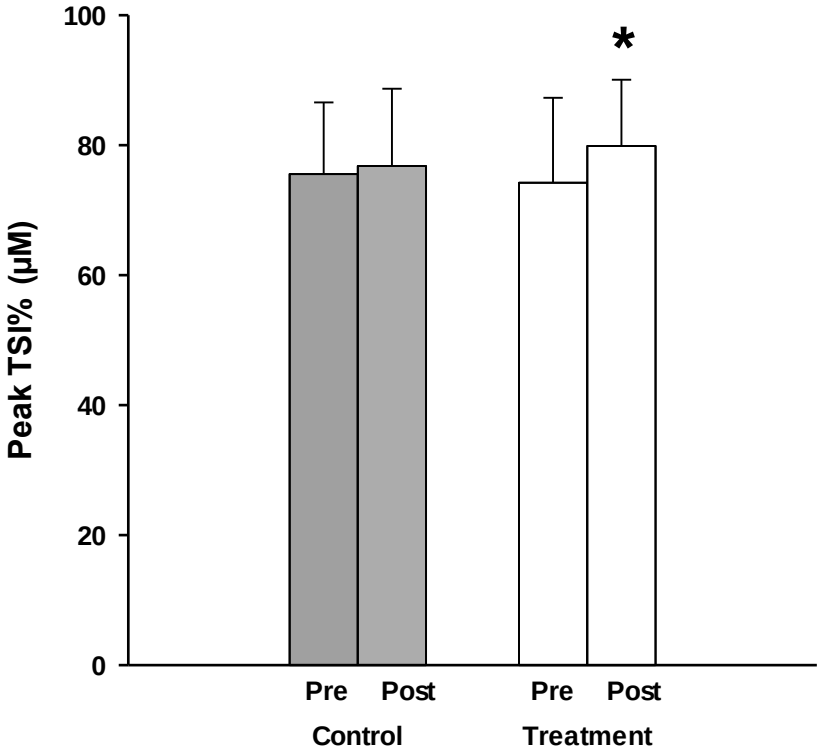


Figure 9 – Peak TSI% following occlusion. * Significant versus pre. P < 0.05. (mean ± SD)

Time-to-Peak NIRS Results

Time to peak Post intervention TSI% values remained unchanged in both legs compared to Pre-Intervention (P=0.12). No significant changes were found between Post-intervention and Pre-Intervention in the remaining time-to-peak parameters (time to peak [Hb] total (P=0.10)), time-to-peak [HbO_2] (P=0.11), time-to-peak [HHb] (P=0.89)) in any of the studied legs.

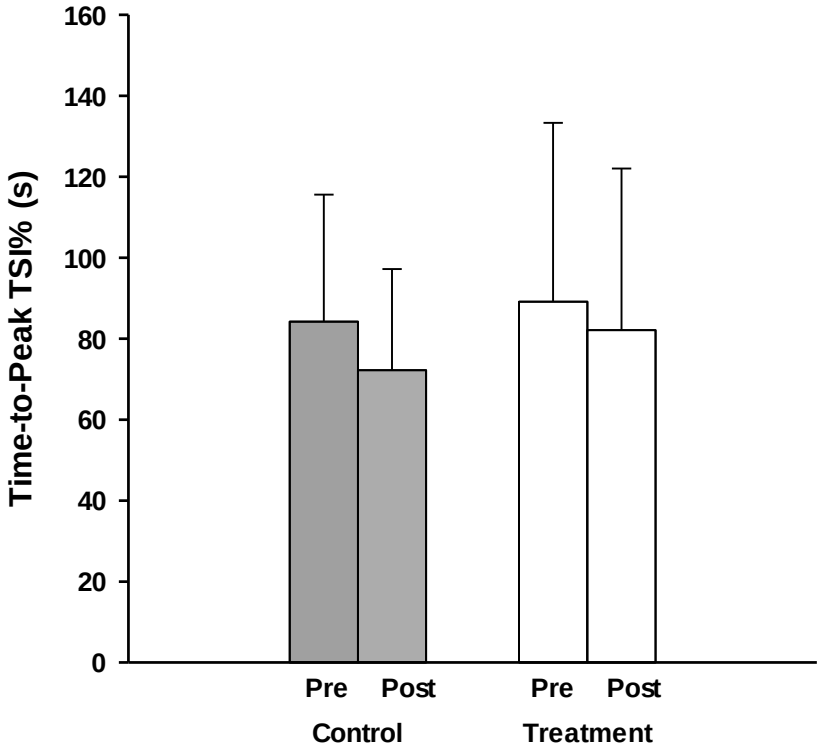


Figure 10 – Time-to-Peak TSI% following cuff occlusion.

Chapter 5: Discussion

The primary findings of the present study were that (1) similar to previous work, the shear rate treatment via a blood pressure cuff increased retrograde shear measured in the superficial femoral artery; (2) following acute increases of retrograde shear stress, resting TSI% was higher the treatment leg but not control; (3) post-intervention [Hb]total and [HbO_2] resaturation rates and peak TSI% in the treatment leg, but not control leg, were significantly increased compared to pre-intervention. These results are at odds with our hypothesis that retrograde shear treatment would affect negatively microvascular function by decreases in the rate of resaturation, time-to-peak resaturation, and peak saturation. Microvascular function is really relevant because it allows an indispensable exchange of gases (primarily CO_2, O_2) and substances (metabolic byproducts and substrates) to move from muscle to the blood and vice versa.

In line with our study, the impact of retrograde and oscillatory shear on endothelial function has previously been studied in both animal and cell culture models. Chappell et al. (1988) showed how human umbilical vein endothelial cells (HUVECs) were capable of discriminating between flow types, being differently affected by oscillatory flow and shear, which consists of a high retrograde shear. The endothelium layers were exposed for 24 hours to a very low mean shear stress with oscillatory shear stresses between +5 and –5 dynes/cm^2. Increase adhesion of monocytes and special morphology (polygonal morphology versus elongated and alignment morphology when exposed to high laminar steady shear) was observed after oscillatory flow exposure. This morphologic adaptation was also reported in confluent bovine aortic endothelial cells (BAECs) [4]. In BAECs and murine aortic endothelial cells (MAECs), McNally et

al. (2003) showed how oscillatory shear increased highly superoxide anion ($\cdot O^{2-}$) production compared to static and laminar conditions, which is suggestive of an increases in reactive oxygen species (ROS) [5]. In agreement with McNally, Hwang et al. (2003) also stated that oscillatory flow enhanced oxidative stress by increases in O^{2-} production, in turn, enhancing LDL oxidation and upregulating inflammatory markers in BAECs [6]. In this type of cells as well, Ziegler et al. (1998) demonstrated that oscillatory flow was responsible for the increase in endothelin-1 (ET-1) and decrease in nitric oxide (NO), leading to endothelial dysfunction [7]. These studies set a background about the underlying physiological mechanisms of retrograde and oscillatory shear on endothelial cells in vitro. Unfortunately, not all conclusions from in vitro studies can be directly applied to the intact circulation.

Recently, several studies have reported changes also in macrovascular endothelial function following exposure to retrograde or oscillatory shear stress in the intact human circulation. Thijssen et al. (2009) demonstrated an indirect dose- response relationship between the 30 min application of retrograde shear and endothelial function, measured by endothelial-dependent flow mediated dilation (FMD), in the brachial artery of young men [8]. The duration of the shear rate intervention and the magnitude of the retrograde shear was similar that used in the present study. Similarly, Totosy et al. (2015) reported results in spinal cord patients that an increased oscillatory shear index (due to increases in retrograde shear) acutely impaired FMD% in the brachial artery and superficial femoral artery (SFA) [9]. Totosy used the same retrograde shear intervention protocol as we used. However, unlike the results of Thijssen et al. (2009) and Totosy et al (2015), we did not observe decreases in vascular

function following the shear rate intervention. This may be due to the location along the arterial tree in which measurements were performed. Both Thijssen et al. (2009) and Totosy et al (2015) evaluated macrovascular function at the large brachial and femoral arteries respectively. In the present study, we evaluated the effects of acute retrograde shear on microvascular reactivity measured via NIRS. Our divergent findings may be due to different retrograde shear patterns elicited along the arterial tree by our cuff intervention (i.e. since the surface/volume relationship is higher in smaller vessels, adhesion forces between the blood and the wall of the vessels become significant, having to exert more negative pressure to produce the enough retrograde shear to see the same effects than in a big blood vessel). Another potential limitation could be the limited effect of the retrograde shear on microvascular function, which entitles either a ceiling effect and/or no effect due to the 5 min rest period after the retrograde shear treatment; NIRS limitations have to be considered also. Besides, we did not measure FMD response after the retrograde shear treatment application so we are not aware whether it was or not impaired, although Thijssen and Totosy have proved it is [8, 9]. Different substances have been identified as factors playing a vasoactive role in the FMD response in the macrocirculation, such as ACE (angiotensin converting enzyme), acetylcholine, angiotensin, endothelin-1, nitric oxide (NO), etc. Special consideration has been given to NO and endothelin-1 since they are thought to be more involved in the reactive hyperemia response of the FMD technique. Recently in 2013, Crecelius et al. have proposed that the activation of both inward rectifying potassium channels (K_{IR}) and Na^+/K^+-ATPase are independent of the production of NO by the endothelium and have been proved to explain nearly all of the total reactive hyperemia response in

humans. [26, 27] This may explain the positive outcomes reached in this study if we assume that FMD was also happening in the microcirculation and NO did not have any positive impact on the microvascular endothelium.

We quantified how many hours a week of vigorous, moderate, light physical activity and strength training the participant did (Appendix C), thinking of a possible protective adaptation mechanism of the vasculature against retrograde shear, although this has not been reported in the literature. 10 out of total 17 participants performed high intensity exercise, with an average of 2.75 h per week per person. 12 participants performed some moderate intensity physical activity, with an average 3.65h per week per person. Every subject walked an average of 5.97h a week. Only 4 did resistance or strength exercise, with an average of 1.83h a week. Pointing this out, we can rule out any possible existent exercise adaptation which could impact our final results, if that would be the case.

Similar to our study, Schreuder et al. (2014), investigating the effect of increasing levels of 30 min retrograde shear on FMD in older males in brachial and superficial femoral arteries, concluded that increases in cuff pressure resulted in increases in retrograde shear, but did not impair FMD [14]. This suggests that the shear rate intervention can have divergent response in different populations.

NIRS results from our study showed an increase in microvascular reactivity after the retrograde shear stress intervention, as demonstrated by increased post-intervention [Hb]total and [HbO2] resaturation rates and peak TSI% in the treatment leg. Microvascular reactivity measured via NIRS to date has primarily been used in clinical populations. De Blasi et al. (2005) evaluated microvascular reactivity in septic

shock patients compared to healthy subjects. They reported that the reperfusion time was longer after vascular occlusion and that the increase in TSI% was lower in the septic patients [11]. In the present study we observed an increase in resaturation rate, which would parallel resaturation time, and an increased TSI. Kragelj et al. (2001) also used NIRS to evaluate microvascular reactivity in patients with peripheral vascular disease. They determined that recovery times after arterial occlusion for HbO_2, HHb, and TSI% were significantly longer in patients versus controls, suggesting that conditions in which microvascular function is impaired the NIRS response post-occlusion is slowed. The findings of De Blasi et al. (2005) and Kragelj et al (2001) allow us to interpret our NIRS derived measurements of microvascular reactivity. In both studies, the clinical population had slower and lower NIRS responses post-occlusion. Therefore, the increased resaturation rate observed in the present study suggests that the retrograde shear rate intervention had at best a positive impact on microvascular reactivity.

Implications and Future Directions

To our knowledge, this is the first research studying the effects of acute retrograde shear on microvascular reactivity via NIRS. Given that all of the studies to date have focused on cell culture or large artery endothelial function, comparing the findings of the present study to previous work is difficult. The lack of a standardized protocol results a methodological problem (e.g. placement of the cuff, duration and intensity of occlusion, population studied), as well as differences in methods of

analyzing the data and in parameters studied, may lead to discrepancies between studies.

Future research is required to better understand retrograde shear and its consequences on pathogenesis of cardiovascular diseases, especially atherosclerosis. It will be of interest to see how different intensities of retrograde shear affect microvascular reactivity, it may be a ceiling effect or an adaptation from the vasculature which suppress the negative effect. Studies could also examine different muscles, it may be a distinct effect between lower and upper body, since the lower body is more prone to develop atherosclerosis. Physical activity may have some effect protecting the vasculature against the effects of retrograde shear, but this is nowadays unknown so it may be interesting to address it in the future.

Chapter 6: Conclusion

This study investigated the impact of the application of retrograde shear on endothelial function at a microvascular level (i.e. arterioles, capillaries, and venules). To our knowledge, this is the first study that has investigated microvascular function and how can it can be impacted by acute changes retrograde shear stress. Microvascular reactivity, via NIRS derived measurements of tissue oxygenation characteristics, was assessed in each participant before and immediately after exposure to a 30 min retrograde shear treatment. Only one leg of the subject was exposed to the shear stress treatment, with the contralateral leg serving as a control leg, which allowed comparisons between legs. In the treatment leg, application of the external cuff resulted in significant increases in retrograde shear stress and an increase in the oscillatory shear index. These increases were not observed in the control leg. Following acute exposure of the increased retrograde shear stress, the NIRS showed a significantly higher resting TSI% post-intervention in the treatment leg that was not observed in the control. Similarly, the post-intervention [Hb]total and [HbO2] resaturation rates and peak TSI% in the treatment leg, but not control leg, were significantly increased compared to pre-intervention. This is at odds with our hypothesis that retrograde shear treatment would affect negatively microvascular function by i) decreases in the rate of resaturation, ii) decreased time-to-peak resaturation, and iii) decreased peak saturation. The data suggests that our intervention in the treatment leg had a positive impact on NIRS derived parameters of microvascular reactivity. Future research may address the mechanisms underlying these findings.

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Appendix A: IRB Outcome Letter

The UNIVERSITY of OKLAHOMA

Institutional Review Board for the Protection of Human Subjects

Initial Submission – Board Approval

Date: April 08, 2016

To: Carl Ade, PhD

IRB#: 6593
Meeting Date: 03/21/2016
Approval Date: 04/07/2016
Expiration Date: 02/28/2017

Study Title: Impact of Acute Increases in Retrograde Shear Rate on Microvascular Function

Reference Number: 649492
Study Status: Active - Open - Expedited
Collection/Use of PHI: Yes

At its regularly scheduled meeting the IRB reviewed the above-referenced research study. Study documents (e.g. protocol, consent, survey, etc.) associated with this submission are listed on page 2 of this letter. To review and/or access the submission forms (e.g. application) as well as the study documents approved for this submission, open this study from the *My Studies* option, click to open this study, look under Protocol Items to click on the current *Application*, *Informed Consent* and *Other Study Documents*.

* **The IRB has determined that this study qualifies for future expedited review under Category 4.**

If this study required routing through the Office of Research Administration (ORA), you may *not begin your study yet*, as per OUHSC Institutional policy, until the contract through ORA is finalized and signed.

As principal investigator of this research study, it is your responsibility to:

- Conduct the research study in a manner consistent with the requirements of the IRB and federal regulations at 45 CFR 46 and/or 21 CFR 50 and 56.
- Request approval from the IRB prior to implementing any/all modifications.
- Promptly report to the IRB any harm experienced by a participant that is both unanticipated and related per IRB Policy.
- Maintain accurate and complete study records for evaluation by the HRPP quality improvement program and if applicable, inspection by regulatory agencies and/or the study sponsor.
- Promptly submit continuing review documents to the IRB upon notification approximately 60 days prior to the expiration date indicated above.

In addition, it is your responsibility to obtain informed consent and research privacy authorization using the currently approved, stamped forms and retain all original, signed forms, if applicable.

If you have questions about this notification or using iRIS, contact the IRB @ 405-271-2045 or irb@ouhsc.edu.

Sincerely,

Karen Beckman, MD
Chairperson, Institutional Review Board

1105 N. Stonewall Avenue, Oklahoma City, OK 73117 (FWA0007961)

Appendix B: Informed Consent

Consent Form
University of Oklahoma Health Sciences Center (OUHSC)
University of Oklahoma, Norman Oklahoma

IMPACT OF ACUTE INCREASES IN RETROGRADE SHEAR RATE ON MICROVASCULAR FUNCTION

Sponsor: Department of Health and Exercise Science
Principle Investigator: Carl. J Ade, Ph.D.

This is a research study. Research studies involve only individuals who choose to participate. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to volunteer for this research study because you are a healthy person between the ages of 18 to 45.

Why Is This Study Being Done?
The purpose of this study is to determine the ability of acute changes in retrograde shear rate (i.e., how your blood moves) on the function of your small blood vessels.

How Many People Will Take Part In The Study?
A maximum of 25 men and women between the ages of 18-45 will take part in this study; all at this location.

What Is Involved In The Study?

Procedures
If you agree to be in this study you will be asked to complete a health history and screening questionnaire prior to beginning the study. Additionally, you will also be asked to complete the following tests on a single visit to the laboratory.
The following test will be performed in the below order.

1. **Anthropometric measurements:** Your height and weight will be measured.

2. **Resting blood pressure:** An average blood pressure will be taken after ten minutes of rest in a supine position. A blood pressure cuff will be inflated three individual times with a minute in-between each measurement.

3. **Pre-Microvascular Function:** Blood pressure cuffs on your legs will be inflated to >250 mmHg for 5 minutes. When five minutes have passed, the cuff will be deflated and measurements of how your small blood vessels react (i.e., microvascular function) will be measured with a non-invasive near infrared spectrometer that shines light onto your skin.

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4. **Retrograde shear treatment:** A blood pressure cuff that is placed on one of your legs will be inflated to the target pressure of 75 mmHg. The leg that we place this cuff on will be randomly chosen (like the roll of dice).

5. **Post-Microvascular Function:** A blood pressure cuff on your leg will be inflated to 220 mmHg for 5 minutes. When five minutes have passed, the cuff will be deflated and measurements of how your small blood vessels react (i.e., microvascular function) will be measured with a non-invasive near infrared spectrometer that shines light onto your skin.

How Long Will I Be In The Study?
You will be in the study for 1 day of experimental testing.

There may be circumstances under which your participation may be terminated by the investigator without regard to your consent. These include your inability to follow study requirements.

You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the investigators first.

What Are The Risks of The Study?
The risks of the blood pressure cuff procedure may cause temporary discomfort which could include some numbing and tingling in your arms.

For more information about risks and side effects, ask the researcher by contacting him directly: cade@ou.edu 24 hours a day 7 days a week.

Are There Benefits to Taking Part in The Study?
If you agree to take part in this study, there is no medical benefit to you. We hope the information learned from this study will benefit the community providing information on the cardiovascular system.

What Other Options Are There?
You may choose not to participate in the study.

What About Confidentiality?
The data collected from the current study will be disseminated to the research community; however no elements in the publication will have identifiers that would link directly to you. All data will be coded and secure during all phases of research.

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

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There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the Department of Health and Exercise Science and the OUHSC Institutional Review Board.

What Are the Costs?
The study sponsor will pay for all costs related to your participation in this study.

Will I Be Paid For Participating in This Study?
You will be compensated with a free T-shirt or $15 gift card upon completion of the study.

What if I am Injured or Become Ill While Participating in this Study?
In case of injury or illness resulting from this study, emergency medical treatment is available. However, you or your insurance company will be expected to pay the usual charge from this treatment. The University of Oklahoma Norman Campus has set aside no funds to compensate you in the event of injury.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. Please be sure to discuss leaving the study with the principal investigator. You may discontinue your participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

We will provide you with any significant new findings developed during the course of the research that may affect your health, welfare or willingness to continue your participation in this study.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.

Whom Do I Call If I have Questions or Problems?
If you have questions, concerns, or complaints about the study or have a research-related injury, the researcher(s) conducting this study can be contacted at (405) 325-8943 (office) during normal business hours or cade@ou.edu 24 hours a day.
If you have any questions about your rights as a research participant, concerns, or complaints about the research and wish to talk to someone other than individuals on the research team or if you cannot reach the research team, you may contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.





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Signature:
By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

PARTICIPANT SIGNATURE (age ≥18)	Printed Name	Date
SIGNATURE OF PERSON OBTAINING CONSENT	Printed Name	Date

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Appendix C: Subject Identification and Health History

I.D. _____ _____ _____ _____
Initials *Subject #*

Study ID: 6593

Date _____/_____/_____

Name_____ **Age**_____ **Date of Birth**_____/_____/_____

Address_____**City**_____**State**_____**Zip**_____

Phone # (_____)_____ **Email** _____

Primary Physician _____ **Last Physical Examination** _____

Emergency Contact _____**Phone #** (_____)_____

If you answer "Yes" to any of the below questions, you will need a physician's approval before testing.

1.)	Has a doctor ever said that you have a heart condition and that you should only do physical activity recommend by a doctor?	Yes	No
2.)	Do you feel pain in your chest when you do physical activity?	Yes	No
3.)	In the past month, have you had chest pain when you were not doing physical activity?	Yes	No
4.)	Do you lose your balance because of dizziness or do you ever lose consciousness?	Yes	No
5.)	Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?	Yes	No
6.)	Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?	Yes	No
7.)	Has a doctor ever said that you have microvascular or peripheral artery disease?	Yes	No
8.)	Has a doctor ever said that you have COPD, asthma, lung disease, or cystic fibrosis?	Yes	No
9.)	Has a doctor ever said that you have Diabetes (Type 1 or 2) or renal disease?	Yes	No
10.)	Do you know of any other reason why you should not do physical activity?		

Have you ever had, or currently have any of the following? (please √)

___Asthma	___Arthritis
___Anemia	___Heart Disease/Heart Attack
___Chest Discomfort/Pain	___Light Headed/Dizziness/Fainting
___Diabetes	___Unusual Shortness of Breath
___Heart Murmur	___Stroke
___Seizures	

If you answered yes or checked (√) any of the above, please explain in detail and list age of onset.

_____
_____
_____

1.) Has your physician ever said you have high blood pressure?	Yes	No
a. If "Yes" is your blood pressure controlled via medication?	Yes	No
2.) You are a male 45 or over or a female 55 or over	Yes	No
3.) Has your physician ever said you have high cholesterol?	Yes	No
a. If "Yes" is your cholesterol controlled via medication?	Yes	No
4.) Do you currently smoke?	Yes	No
5.) Do you have a family history of heart disease? (Heart disease or sudden death before 55 for male first relative and before 65 for female first relative)	Yes	No

Please list any prescribed and/or over the counter medications and purpose for taking them.

_____
_____
_____

Please list any over-the-counter supplements and purpose for taking them.

_____
_____

Females:

1.) Are you pregnant? Yes No
2.) Do you have a regular menstrual cycle? Yes No
3.) Have you experienced menopause? Yes No

Recent Physical Activity History

Think about all the *vigorous* activities which take *hard physical effort* that you did in the last 7 days. Vigorous activities make you breathe much harder than normal and may include heavy lifting, digging, aerobics, or fast bicycling. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities?
   _____ Days per week

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?
   __ __ Hours per day
   __ __ Minutes per day

Now think about activities which take *moderate physical effort* that you did in the last 7 days. Moderate physical activities make you breathe somewhat harder than normal and may include carrying light loads, bicycling at a regular pace, or doubles tennis. Do not include walking. Again, think about only those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities?
   ____ Days per week

4. How much time did you usually spend doing **moderate** physical activities on one of those days?
   __ __ Hours per day
   __ __ Minutes per day

Now think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?
   ____ Days per week

6. How much time did you usually spend **walking** on one of those days?
   __ __ Hours per day
   __ __ Minutes per day

Now think about the time you spent doing resistance/strength exercises in the last 7 days.

5. During the **last 7 days**, on how many days did you **resistance/strength exercises** for at least 10 minutes at a time?

   ____ Days per week

6. How much time did you usually spend **resistance/strength exercises** on one of those days?

   __ __ Hours per day

   __ __ Minutes per day

6. What exercises and limbs did you train?