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Circadian variations in aortic stiffness, sympathetic vasoconstriction, and post-ischemic vasodilation in adults with and without type 2 diabetes.

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Circadian variations in aortic stiffness, sympathetic vasoconstriction, and post-ischemic vasodilation in adults with and without type 2 diabetes – Alessandro Gentilin

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Abstract

The current literature reveals a lack of information on the circadian variations of some important cardiovascular risk factors related to the work of the heart or the capacity to provide blood and oxygen to various tissues. These factors include aortic stiffness, peripheral vasoconstrictor responsiveness, and post-ischemic vasodilation capacity. Furthermore, it is not clear whether the impact of an external stressor capable of activating the sympathetic nervous system could have greater repercussions on the cardiovascular system in the morning than in the evening. Given the higher incidence of acute cardiovascular events in the morning than in the evening, the studies undertaken in this thesis aim to investigate the circadian variations of these factors that are linked to cardiovascular risk, both at rest and during acute activation of the sympathetic nervous system. Type 2 diabetes (T2DM) is a condition that induces deleterious changes in cardiovascular function, impacting cardiovascular mortality and morbidity. Thus, the impact of diabetes will be evaluated. As a secondary purpose, considering the sex differences in the incidence and prognosis of cardiovascular disease, the effect of sex will be evaluated.

Aortic stiffness proved not to be increased in the morning compared to the evening at specific times when the cardiovascular risk is significantly different, both at rest and during sympathetic activation. However, while healthy older women show similar aortic stiffness values compared to their male counterparts during acute stress, older women with T2DM reported greater aortic stiffness compared to men with T2DM. The post-ischemic forearm vasodilation is blunted in the morning compared to the evening in healthy elderly and such an attenuated vasodilation capacity impairs blood flow supply towards the ischemic area. The presence of T2DM does not affect vasodilation capacity and reactive hyperemia, but induces circadian variations in arterial pressure. The peripheral vasoconstriction triggered by a standardized sympathetic stressor is similar between morning and evening, regardless of the presence of T2DM and reduced baseline vascular conductance values in the morning. However, the peripheral vasoconstriction responsiveness is

blunted in individuals with T2DM than in healthy ones as sympathetic activation induces vasodilation on the contralateral forearm in individuals with T2DM and vasoconstriction in healthy age-matched subjects. This finding highlights a neurovascular response to an external stressor altered by T2DM.

Taken together, our findings suggest that the baseline state of constriction of the peripheral vascular tissue is greater in the morning than in the evening, but this fact is not due to greater sympathetic vasoconstriction responsiveness in the morning. Higher morning vasoconstriction at baseline however affects the capacity of a vascular tissue to dilate and, in turn, to supply blood to an ischemic tissue. Similar sympathetic vasoconstriction responsiveness between morning and evening is a likely factor explaining similar or lower values of central artery stiffness in the morning than in the evening, not only at rest but also during sympathetic excitation. Paradoxically, adults with T2DM report an increase in sympathetic-mediated dilatation capacity on the vascular tissue, which might be a defense mechanism that allows to reduce the central pressor response during sympathetic excitation.

CHAPTER 1: GENERAL INTRODUCTION

ANATOMY OF THE AUTONOMIC NERVOUS SYSTEM

The nervous system is anatomically divided into central and peripheral nervous system [1, 2]. The central nervous system includes brain and spinal cord. The peripheral nervous system includes ganglia and nerves located outside of the central nervous system. The peripheral nervous system is in turn divided into an afferent part that transduces peripheral information and sends it up to the higher centers; a somatic efferent portion that is responsible for the voluntary control of movements; and an efferent portion, named autonomic nervous system, that modulates basic functions that do not require conscious involvement of the brain. The autonomic nervous system can be further divided into sympathetic, parasympathetic, and enteric nervous system.

The sympathetic nervous system is composed by preganglionic neurons that are located inside the intermedium-lateral-column of the thoracic and lumbar spinal cord [3–5]. Neuron axons travel across the nervous anterior-root, reaching their analogous spinal nerves. These fibers achieve their reciprocal spinal ganglia in the sympathetic chain by traveling through the white-ramus branch and arrange synapses with sympathetic postganglionic neurons, upper or lower ganglia of the same chain, or other sympathetic peripheral ganglia. Finally, postganglionic fibers reach their target organs. A peculiarity of the sympathetic nervous system is that sympathetic ganglia are located far from their target organs and composed of short-caliber, lower-speed nervous transmission C-type fibers [1–4]. A portion of sympathetic fibers also connect with the adrenal medulla [1–4]. These fibers do not arrange synapses inside the thoracic ganglia chains, but with the neurosecretory-cells in the adrenal medulla through the splanchnic nerves. Sympathetic stimulation of the adrenal medulla leads to a release of catecholamines into the bloodstream. The sympathetic nervous system can thus modulate its activity through both nervous and secretory pathways.

The parasympathetic nervous system works is composed by preganglionic and postganglionic neurons, which are mostly placed close to or just over the target organ [1–4, 6]. Preganglionic neurons are located in the cervical and sacral region of the spinal cord and then connected, without being interrupted, to their target postganglionic neurons through long nervous fibers. Short axons between 1 millimeter and few centimeters long are then directed inside the target tissue. Approximately 75% of the parasympathetic fibers are found within the vagus nerve, which reaches abdominal and thoracic organs. Due to the different anatomy, the sympathetic nervous transmission is this slower than the parasympathetic one. Moreover, sympathetic stimulation mostly provides systemic effects, while parasympathetic stimulation provides organ-specific responses because of the shorter postganglionic fibers located just over the target organ.

Although the cardiovascular system is innervated by both autonomic branches, the innervation of the heart and arterial vessels differs [1–4]. The heart receives both the sympathetic and parasympathetic innervations, which affect the cardiac frequency and contractility by changing the spontaneous activity of the cardiac pacemaker cells [5]. In contrast, arterial vessels are innervated by adrenergic fibers only [4]. Arterial vessel neural constriction is therefore related to the average sympathetic discharge. The sympathetic tone keeps arterial vessels in a partial constriction state, sending low-frequency impulses between 0.5 and 2 hertz. The sympathetic tone is modulated by several brain structures, including the hypothalamus, which can exert powerful excitatory and inhibitory effects [1–4]. The vasomotor activity is controlled by the solitary tract nucleus in the brainstem [1, 5]. The solitary tract nucleus is the arrival point of all cardiovascular afferent information. It receives information from baroreceptors through the vagus and glossopharyngeal nerves, as well as from the periphery through spinal nerves. The solitary tract nucleus arranges connections with different areas of the brainstem and brain through internal nerve fibers. The predominant sympathetic nuclei are the ventrolateral caudal medulla and the ventrolateral rostral medulla [1, 5]. These structures are located in the brainstem and connected to the solitary tract nucleus. The ventrolateral caudal medulla is connected to the solitary tract nucleus through mono-synaptic fibers and contains both inhibitory and excitatory inter-neurons

connected to the ventrolateral rostral medulla. This structure is involved in the baroreceptor reflexes [2]. The ventrolateral rostral medulla, or vasomotor center, is the main structure that generates the sympathetic nervous activity [1, 5]. It receives information from several inhibitory mono-synaptic neurons of the ventrolateral caudal medulla and transmits its response to the sympathetic preganglionic neurons via several excitatory mono-synaptic axons. At that point, fibers are directed to the ganglia of the sympathetic chain. The predominant parasympathetic nuclei are the nucleus ambiguous and the vagal dorsal-motor nucleus [1, 5, 6]. These structures also connect to the brainstem. The nucleus ambiguous is composed by several motor-neurons placed in the reticular formation. The external portion contains parasympathetic cardio-inhibitory neurons directed to the heart [1, 5, 6]. The nucleus ambiguous also sends efferent fibers to the esophagus, pharynx, and larynx. The vagal dorsal-motor nucleus has parasympathetic fibers predominantly directed to the gastrointestinal tract and lungs, and just a little amount directed to the heart. Therefore, the parasympathetic control of the heart mainly comes from the nucleus ambiguous and just to a minor extent from the vagal dorsal-motor nucleus [1, 5, 6].

AUTONOMIC NEUROVASCULAR REGULATION

Vascular conductance is a physiological parameter that conceptually expresses a mean vascular section through which blood flows at a given pressure difference [7]. This parameter expresses the ease with which blood flows through a vascular bed as the (average) section of the vessel is inversely related to the resistance that the blood encounters while flowing in accordance with Poiseuille's law [8]. The precise regulation of vascular conductance is a long-lasting interest in physiology as this mechanism is essential for the regulation and maintenance of blood pressure and thus adequate delivery of blood flow and oxygen to vital tissues to sustain life [7]. Indeed, blood flow through a vascular tissue is determined by the systemic arterial pressure times vascular conductance. The control of vascular conductance is complex and is dynamically regulated by a balance between sympathetic nervous system vasoconstriction and local endothelial vasodilation factors [7]. Norepinephrine released from sympathetic nerve endings and adrenal glands binds to post-synaptic α-adrenergic receptors on vascular smooth muscle to increase

vasoconstriction by promoting Ca2+ influx [9]. Epinephrine released from the adrenal grand can bind to both β2-adrenoceptors, inducing vasodilation, and postjunctional α1- or α2-adrenoceptors, inducing vasoconstriction (more details below) [9]. In contrast, nitric oxide is the main endothelial vasodilator factor. It can induce vasodilation by inhibiting Ca^{2+} influx and decreasing free $[Ca2+]_I$ [10] via activation of guanylate cyclase in smooth muscle cells and probably by stimulating the release of vasodilator prostaglandins from endothelial cells [11]. Other local vasodilator factors can affect vascular conductance. Adenosine has been suggested to be responsible for 20 to 40% of the maintained phase of muscle vasodilatation following muscle contraction by binding to A_{2A} receptors on arterial smooth muscle [12]. Increased levels of extracellular potassium have also been suggested to induce hyperpolarization and consequent relaxation of vascular smooth muscle via Na+/K+-ATPase pump stimulation [11, 13]. Indeed, the membrane potential of vascular smooth muscles regulates the open-state probability of voltage-gated Ca2+ channels and consequently Ca2+ influx. It is well established that ATP binds to purinergic (P2Y2) receptors on the endothelium to evoke vasodilation through the downstream production of nitric oxide and prostaglandins, and activation of K+ channels that hyperpolarize vascular smooth muscle [12]. Hydrogen ions can relax vascular smooth muscle by diminishing the intracellular Ca2+ concentration [11]. Oestrogen has a relaxant effect on the vascular smooth muscle and has been shown to augment vasodilation [14–16]. Our current understanding of how the control mechanisms interact and are integrated to precisely control vascular conductance and blood flow is limited. Moreover, the previous investigations have mainly focused on the blunting of flow-mediated vasodilation of the brachial artery. This variable is related to cardiovascular risk but does not provide information on the precise regulation of blood flow and oxygen delivery to the limb. Furthermore, the interaction between the various factors may differ between central arterial and muscular segments due to a different amount of collagen, vascular smooth muscle, and adrenergic receptors. However, the study of the neurovascular control has mainly focused on muscle arteries and little is known on central arteries such as the aorta.

EFFECTS OF ACUTE SYMPATHETIC ACTIVATION ON THE VASCULAR TISSUE

The sympathetic nervous system can be activated by many stimuli, including physical stressors, mental stress and emotions [17, 18]. The effect of different types of sympathetic stimulants on neurovascular and neuroendocrine regulation is not universal [19–22] as these may involve different physiological and cognitive mechanisms. Effects attributable to alpha-adrenergic activation by using physical stressors and beta-adrenergic activation by using mental stress have been reported [19, 21–23]. Physical stressors generally induce sympathetic activation via mechanical, thermal, or chemical stimulation of peripheral receptors and central reflex cardiovascular responses in the brain stem [2]. In contrast, sympathetic activation via mental stress and emotions can occur without physical stimulation of peripheral receptors, but requires attention to select meaningful information from the environment and to link it with appropriate emotional responses [20] and involves the activation and stimulation of a complex of brain structures including the amygdala and hypothalamic-pituitary-adrenal (HPA) axis [2]. Different neurovascular effects between physical and mental stressors are consistent with a different level of sympathetic activation, as well as with a different type and quantity of neurotransmitters released and their specific effects on alpha- and beta-adrenoreceptors [2, 21, 22]. The study of stress-induced cardiovascular responses might provide insight into future cardiovascular health status. Indeed, previous studies have found that stress-induced exaggerated cardiovascular response at a younger age might predict future blood pressure status in later life [21].

Although each sympathetic stressor induces slightly different vascular responses [19], acute sympathetic activation predominantly induces a release of norepinephrine from sympathetic nerve endings and adrenal glands, which binds to post-synaptic α-adrenergic receptors on vascular smooth muscle to increase vasoconstriction by promoting Ca^{2+} influx [9]. Epinephrine released from the adrenal grand has a higher affinity for β2- than postjunctional α1- or α2-adrenoceptors. Low concentrations of epinephrine can bind to β2-adrenoceptor, inducing vasodilation. In contrast, high concentrations of epinephrine can also bind to α1- and α2-adrenoceptors, overpowering the vasodilatory effects of β2-

adrenoceptor stimulation and resulting in vasoconstriction. The sympathetic nervous system nerve endings can also release other neurotransmitters with different effects on the vascular tissue. It is now recognized that non-adrenergic neurotransmitters adenosine-5'-triphosphate (ATP) and neuropeptide Y (NPY) released from sympathetic nerve endings contribute to sympathetic vasoconstriction by binding to purinergic P2X and NPY Y^1 receptors on the vascular smooth muscle, respectively [24, 25]. It has been shown that the type, pattern and quantity of neurotransmitter release is dictated by the frequency of neuron firing [24, 25]. In particular, low discharge frequencies favor ATP release followed by NE, whereas mid-range discharge frequencies produce both ATP and NE release, while high discharge frequencies favor NPY release [24, 25]. Although the sympathetic nervous system can potentially induce both vasoconstriction and vasodilation, vasoconstrictive effects are predominant and acute sympathetic activation results in vasoconstriction. Consistent with this notion, acute sympathetic activation has been shown to blunt the normal nitric oxide-mediated vasodilation of the brachial artery after a brief period of ischemia to a different extent according to the sympathetic stressor employed [19, 23, 26]. However, the current knowledge on how sympathetic stressors affect vascular conductance and consequently blood flow is limited.

CIRCADIAN VARIATIONS IN THE NEUROVASCULAR REGULATION

Daily variations in the environment lead organisms to organize behaviors with activity/rest periods that route the bright/dark pattern [27, 28]. Such rhythmicity lets organisms forecast and synchronize their physiological functions to recurring daily environmental variations. Increasing evidence has suggested that intrinsic circadian clocks are closely associated with cardiovascular functions [29, 30]. A 24-hour circadian clock in the central autonomic outflow towards the heart and vascular tissue has been extensively accepted. The day-night pattern in the autonomic nervous system function is strictly associated with the circadian rhythm in healthy individuals and several pathological states [31]. In humans, epinephrine, norepinephrine, and cortisol level, as well as their reactivity, follow circadian rhythmicity [32–35]. Besides the 24-hour central cardiovascular regulation pattern

due to the central clock located in the suprachiasmatic nucleus of the hypothalamus, it is now established that the clock in each tissue or cell works as a peripheral clock and has its roles in controlling the responsiveness of each peripheral organ [36]. In this respect, emerging data have suggested pacemaker signaling within the cardiac tissue itself is crucial in circadian signaling [37]. Furthermore, a peripheral circadian rhythm in several vascular functions has been presented, including endothelium-dependent vasodilatory function and vascular reactivity [27, 28, 36]. There is substantial evidence from animal models and epidemiological studies indicating that disturbance of circadian rhythms is a powerful risk element for a cardiovascular disorder and that the occurrence of cardiovascular disease may have a time-dependent effect [38].

With regard to neurovascular control, several studies have shown that the flow-mediated dilation of the brachial artery decreased in the morning compared to the evening [28]. The activity of the sympathetic nervous system evaluated by measuring the circulating catecholamines also follows a circadian variation. Noradrenaline shows a maximum peak around 12.00 am and a minimum around 02.00 am and it is approximately similar in the early morning compared to evening [39]. Adrenaline shows a maximum peak around 5.00 pm and a minimum peak around 4.00 am, but it is higher in the morning than in the evening [39]. Epinephrine levels have no relationship to sleep or posture, whereas norepinephrine levels are higher with upright posture and while awake than asleep [39]. Circulating cortisol can also decrease endothelial function. Cortisol shows a maximum peak at approximately 06.00-08.00 and a minimum peak at approximately 02.00, and is significantly higher in the morning than in the evening. However, it is not known whether such a circadian hormone influences the control of vascular conductance between morning and evening. Furthermore, it is not known whether the sympathetic neurovascular reactivity is greater in the morning than in the evening in response to the same stressful stimulus.

INCIDENCE OF ACUTE CARDIOVASCULAR EVENTS AT DIFFERENT TIMES OF THE DAY AND POTENTIAL ACTORS

Circadian clocks have been shown to be associated with cardiovascular functions [40, 41]. The incidence of stroke, myocardial infarction, arrhythmia, and sudden cardiac death is higher in the morning compared to the evening [40, 41]. A 40% higher risk of heart attack, a 29% increased risk of cardiac death, and a 49% increased risk of stroke have been suggested in the early morning between 6am and 12am [41]. A role for the sympathetic nervous system has also been argued [42, 43]. Previous findings may support the notion of a greater sympathetic tone in the morning. Arterial pressure and vascular resistance of the forearm have been shown to be augmented in the early morning [44]. Rapid eye movement–related augmented sympathetic activation has been suggested to be particularly evident in the early morning [45, 46]. Endothelial vasodilation function, which can be restrained by the sympathetic outflow [19], has been shown to be blunted in the early morning [45, 47]. Augmented sympathetic vasoconstriction can increase arterial and pulse pressure, increasing the risk for cardiovascular events. Indeed, such effects augment the afterload, work, and oxygen demand of the heart and predispose to vascular lesions [44, 48]. There are many factors strongly and closely related to acute cardiovascular events and sympathetic nervous system-dependent that have yet to be studied. These factors include aortic stiffness, peripheral vasoconstrictor responsiveness, and vasodilation capacity.

EFFECTS OF AGING ON THE CARDIOVASCULAR REGULATION

The preponderance of studies suggests that the tone of the sympathetic nervous system increases with aging. Indeed, healthy elderly people show a higher level of circulating catecholamines and muscle sympathetic nerve activity than healthy young individuals [49–53]. In contrast, NO bioavailability decreases with aging [49–51]. Healthy elderly show a diminished endothelial-dependent vasodilation compared to young healthy individuals. Moreover, there is substantial evidence suggesting several aging-related changes, including altered adrenergic responsiveness for β-adrenergic receptor (vasodilation), β-adrenergic receptor

density reduction, and β-adrenoceptor-G-protein(s)-adenylyl cyclase system abnormalities [54]. The control of vascular conductance is a continuous balance between local vasodilatory effects and sympathetic vasoconstriction [7]. An increase in sympathetic tone along with a decrease in vasodilation capacity as consequence of aging could shift the control of vascular conductance towards a vasoconstrictor dominance. This could potentially impair the circulation of tissue blood flow and consequently the capacity to distribute blood and oxygen to the various tissues. However, these questions still need to be addressed.

EFFECTS OF TYPE 2 DIABETES ON THE CARDIOVASCULAR REGULATION

The rate of cardiovascular events increases in the presence of type 2 diabetes (T2DM) [40, 55, 56]. High blood sugar and insulin in type 2 diabetics have been established to determine early-functional changes and dynamic anatomic remodeling of autonomic pathways controlling the circulation, affecting cardiac and vascular cellular targets and feedback baroreceptor system sensitivity [57–59]. Acute and chronic high blood insulin level has been shown to raise sympathetic dominance, plasma catecholamines, and efferent sympathetic drive to the heart in type 2 diabetes [60–62]. Endothelial vasodilation has been shown to be blunted in individuals with diabetes compared to their healthy counterpart. Neuropathy and symptoms of postural hypotension in individuals with T2DM suggest that the effects of T2DM affect the sympathetic branch of the autonomic nervous system [59, 63]. T2DM augments sympathetic dominance [59, 63, 64]. Subjects with T2DM exhibit augmented sympathetic transduction to blood pressure compared to healthy controls [65], which is strictly connected to peripheral vasoconstriction in response to sympathetic stressors. Besides central autonomic changes, diabetes leads to further deleterious organ adaptations [66]. Cardiac sinus node desensitization has been identified in diabetics [67]. Diabetes-induced remodeling has been shown in vascular tissue and associated with acute endothelial dysfunction, higher arterial stiffness, vascular hypertrophy in small arteries, and impaired responsiveness of vascular smooth muscle stimulants [68, 69]. Such central and peripheral diabetes-induced cardiovascular changes have been proposed

to potentiate sympathetic vasoconstrictor responsiveness and weaken the circadian rhythm of heart rate and blood pressure, leading to a high incidence of hypertension, myocardial infarction, hospitalization, and death in the first 2 years [70–72].

An emerging complication of diabetes is the blunting of the normal circadian rhythm (24-hour cycle) in the heart rate and blood pressure, contributing to hypertension, acute cardiovascular events, and mortality [73–75]. This diabetes-induced dysregulation is overwhelmingly attributed to impaired autonomic nerve input to the heart and vascular tissue associated with altered end-organ autonomic responsiveness. Wide evidence has proposed such a circadian clock disruption to alter the autonomic outflow towards the heart and vascular tissue and raise the reactivity of the sympathetic nervous system to behavioral stressors [32]. The normal circadian rhythm-driven sympathetic amplified outflow in the morning may promote the changeover from sleep to daily life, but excessive sympathetic outflow associated with excessive tissue sympathetic responsiveness are potentially threatened in individuals prone to adverse cardiovascular events [76]. Defining how these adaptations result in alterations in the control of vascular conductance, as a key factor in regulating blood flow towards tissues and consequently the supply of oxygen, is a question not yet addressed.

SEX DIFFERENCES ON THE NEUROVASCULAR REGULATION

Sex differences in the autonomic regulation of the heart, blood pressure, and circulation have been widely suggested [77–80]. Epidemiological studies have consistently identified clear differences in the prevalence and severity of cardiovascular disease between men and women [81, 82]. The cardiovascular pathophysiological disease has been noted to have a different symptomatology and response to treatment in one biological sex compared to the other [81, 82]. Diabetes has been suggested to raise the risk of heart disorder in women more than it does in men because women with diabetes more often have added risk elements, such as obesity, hypertension, and high cholesterol [83, 84]. Although women generally develop heart disease about 10 years later than men, diabetes has been noted to abolish that advantage. In women who have previously had a heart attack, diabetes doubles the risk for a second heart attack and raises the risk for heart failure. Despite

such sex differences, most of the previous literature has been focused on males [85]. The investigation of sex differences in neurovascular modulation may lead to new insights into cardiovascular medicine and prevention. The identification of factors affecting vascular health in one sex may lead to translational research and development of therapeutics applicable to the opposite sex, as well as to differentiated therapies between sexes [86]. The study of sex differences is thus significant for translating findings from basic science to clinical outcome and in developing personalized medical plans [87].

MAIN OBJECTIVES OF THE PHD PROJECT

The current literature reveals a lack of information on the circadian variations of some important cardiovascular risk factors related to the work of the heart or the capacity to provide blood and oxygen to various tissues. These factors include aortic stiffness, peripheral vasoconstrictor responsiveness, and vasodilation capacity. Furthermore, it is not clear whether the impact of an external stressor capable of activating the sympathetic nervous system could have greater repercussions on the cardiovascular system in the morning than in the evening.

Given the higher incidence of acute cardiovascular events in the morning than in the evening, the primary objective is to assess whether the impact of various cardiovascular factors potentially linked to cardiovascular risk is greater in the morning than in the evening. In particular, such evaluations will be performed at rest and in the presence of a standardized, external stressor capable of activating the sympathetic nervous system. Considering that cardiovascular events occur more frequently in adults with compared without type 2 diabetes, the impact of diabetes will be evaluated. As a secondary purpose, considering the sex difference in the incidence and prognosis of cardiovascular disease, which worsens to a greater extent in women with diabetes, the sex differences will be evaluated.

OBJECTIVE 1

Aortic stiffness affects the afterload, work, and oxygen demand of the heart and is a strong predictor of cardiovascular risk. The first objective is to evaluate whether aortic stiffness is increased in the morning versus the evening, at rest and during acute activation of the sympathetic nervous system, in adults with and without type 2 diabetes. It is hypothesized that aortic stiffness increased at morning both at rest and during sympathetic activation.

OBJECTIVE 2

Peripheral vasoconstriction affects pulse pressure and arterial stiffness, and thereby the risk for cardiovascular events by augmenting the afterload, work, and oxygen demand of the heart and the formation of vascular lesions. The second objective is to assess whether peripheral vasoconstriction triggered by a standardized

sympathetic stressor is augmented in the morning versus the evening, in adults with and without type 2 diabetes. It is hypothesized that peripheral vasoconstriction is increased in the morning compared to the evening.

OBJECTIVE 3

The vasodilation capacity following tissue ischemia is a vital function as it allows to supply blood and oxygen towards the ischemic area. The third objective is to evaluate whether vasodilation capacity following tissue ischemia is attenuated in the morning compared to the evening, in adults with and without type 2 diabetes. It is hypothesized that the vasodilation capacity is blunted in the morning compared to the evening and that such an attenuation impairs blood flow towards the ischemic area.

CHAPTER 2: CIRCADIAN AND SEX DIFFERENCES IN CAROTID-FEMORAL PULSE WAVE VELOCITY IN ADULTS WITH AND WITHOUT TYPE 2 DIABETES AT REST AND DURING SYMPATHO-EXCITATION.

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Keywords: Aging; Diabetes mellitus; Arterial stiffness; Cardiovascular disease; Risk factors; Sympathetic activation; Sex differences; Circadian changes.

ABSTRACT

The incidence of cardiovascular events is higher in the morning compared to the evening and differs between sexes. We tested the hypothesis that aortic stiffness, a compelling cardiovascular risk factor, is increased in the morning compared to the evening in adults between 50-80 years either healthy (H50-80) or with type 2 diabetes (T2DM50-80). Sex differences were also investigated. Carotid-femoral pulse wave velocity (cf-PWV) assessed via Doppler Ultrasound, blood pressure, and heart rate were collected at 6am and 9pm, at rest and during acute sympathetic activation triggered by handgrip exercise. Cf-PWV values were lower in the morning compared to the evening in both groups ($p<0.02$) at rest, and similar ($p>0.52$) during sympathetic activation. At rest, cf-PWV values were higher in women compared to men ($p<0.03$). During sympathetic activation, the cf-PWV was similar between sexes in H50-80 ($p=0.20$) and still higher in women in T2DM50-80 ($p=0.006$). These data do not support the hypothesis that aortic stiffness is increased in the morning compared to the evening in both groups, in rest and sympathetic activation conditions. There are differences between sexes, which vary according to the presence of a stressful stimulation and diabetes status. In particular, aortic stiffness is higher in older women compared to men with diabetes during acute stress.

INTRODUCTION

Circadian variations in physiological functions allow organisms to provide adequate physiological responses to recurring daily needs [40]. Circadian clocks have been shown to be associated with cardiovascular functions [40, 41]. Interestingly, the incidence of stroke, myocardial infarction, arrhythmia, and sudden cardiac death is higher in the morning compared to the evening [40, 41]. A 40% higher risk of heart attack, a 29% increased risk of cardiac death, and a 49% increased risk of stroke have been suggested in the early morning between 6am and 12am [41]. Endothelial function, an important index of cardiovascular risk, is blunted in the early morning compared to the evening [45, 47], while peripheral vascular resistance and blood pressure (BP) are increased [44]. Augmented sympathetic activation in the morning may be a cardiovascular risk contributor [44, 47]. Interestingly, overt sex differences in the prevalence and severity of cardiovascular disease, as well as in sympathetic neurovascular modulation, have been documented [86, 88, 89]. Cardiovascular disease has different symptomatology and response to treatment in one sex compared to the other [88]. The incidence of acute cardiovascular events is higher in men compared to women in the reproductive age, however, this trend starts reversing after menopause [15].

Among the possible contributors to the different incidence of acute cardiovascular events in the morning compared to the evening, as well as between sexes, a greater aortic stiffness might be involved. Aortic stiffness is a compelling predictor of all-cause mortality [90]. Aortic stiffness is an independent predictor of fatal stroke in patients with essential hypertension [91]. Circadian variations of carotid-femoral pulse wave velocity (cf-PWV), the gold-standard measure to assess aortic stiffness, have been documented [47, 92, 93]. Augmented aortic stiffness increases the afterload, work, and oxygen demand of the heart, as well as increases BP and pulse pressure [48]. Augmented sympathetic outflow to the heart and blood vessels in the morning may also increase the risk of acute cardiovascular events by augmenting cardiac afterload and pulse pressure, and by reducing baroreflex sensitivity [44, 48].

The incidence of acute cardiovascular events normally increases with aging. The presence of type 2 diabetes (T2DM), however, increases such an occurrence

[40, 55]. T2DM leads to changes in central autonomic control and deleterious organ adaptations [55, 59, 66]. High blood sugar and insulin in individuals with T2DM determine early-functional changes and remodeling of autonomic pathways controlling circulation, affecting cardiac and vascular cellular targets and feedback baroreceptor system sensitivity [55, 58, 59]. Acute and chronic high blood insulin levels in individuals with T2DM augment sympathetic dominance, plasma catecholamines, and efferent sympathetic drive to the heart [59, 63]. Imbalanced autonomic outflow towards the heart and vascular tissue has been associated with several pathological states, including cardiac autonomic neuropathy and deleterious cardiac remodeling in individuals with T2DM [63, 72]. T2DM-induced remodeling has been found in vascular tissue and associated with augmented arterial stiffness, acute endothelial dysfunction, vascular hypertrophy in small arteries, and impaired responsiveness to vascular smooth muscle stimulants [68, 69]. T2DM-induced changes in the cardiovascular system have been suggested to blunt the normal circadian rhythms of heart rate (HR) and BP, leading to a high incidence of hypertension, myocardial infarction, hospitalization, and death [63].

This study aims to compare circadian and sex differences in aortic stiffness in young healthy individuals, old healthy individuals, and old individuals with T2DM, at rest and during sympathetic activation. As the incidence of acute cardiovascular events is higher in the morning compared to the evening, it is hypothesized that aortic stiffness, assessed via cf-PWV, is greater in the morning compared to the evening. Moreover, it is hypothesized that cf-PWV is lower in young women compared to young men and that such sex differences disappear in the older groups. The endothelial function is blunted at 6 am compared to at 9 pm, suggesting increased cardiovascular risk at that morning time [45]. Therefore, the cf-PWV assessment in our study has been performed according to such a timing schedule.

MATERIALS AND METHODS

The cf-PWV assessment was performed on 60 participants. The subjects were a random sample of the population of Northern Italy and were enrolled

through recruitment flyers scattered around the cities. Subjects were divided into 3 groups as follows: 30 healthy individuals from 50 to 80 years old (H50-80), and 30 individuals with T2DM from 50 to 80 years old (T2DM50-80). All groups were sex balanced.

	H50-80		**T2DM50-80**		***H50-80 vs T2DM50-80***
	Men	*Women*	*Men*	*Women*	
Age (years)	66.1(7.1)	66.9(7.4)	67.3(7.5)	66.5(8.1)	p=0.964
Weight (Kg)	76.5(10.0)	70.3(9.4)	86.1(14.9)	79.4(22.0)	p=0.101
Height (m)	1.71(0.05	1.61(0.05*	1.77(0.2	1.59(0.07*	p=0.805
BMI (Kg/m^2)	26.2(2.9	27.1(4.2	27.6(5.2	31.2(7.7	p=0.159
#Systolic BP (mmHg)	136.9(9.4)	128.6(13.2)	144.4(13.7)	140.2(11.4)	p=0.02
#Diastolic BP (mmHg)	81.0(4.6)	78.1(6.9)	82.4(8.9)	82.6(8.9)	p=0.34
#HR (bpm)	62.2(8.5)	59.6(8.2)	63.5(7.2)	68.0(6.0)	p=0.13
#cf-PWV (m/s)	7.7(1.0)	8.8(1.2)*	7.8(0.9)	8.9(1.4)*	p<0.001

*Table 1. Characteristics of the subjects (n=30, sex balanced within each group; * compared to men; # average value between morning and evening measure at rest). Data are reported as mean(SD).*

All participants met common inclusion (50-80 years old) and exclusion criteria (chronic hypertension, atrial fibrillation, cardiac valve disease, not in sinus rhythm, pacemaker-dependent, known significant carotid or femoral artery stenosis, impalpable arterial pulse at site of measurement, use of beta-blockers and ACE-inhibitors, pregnancy or presumed pregnancy). Specific inclusion criteria for

healthy subjects consisted in having fasting blood glucose lower than 100 mg / dL and being free of any cardiovascular, metabolic, neurological, and respiratory disease. Specific inclusion criteria for subjects with T2DM consisted in having been diagnosed with T2DM for at least 1 year. Specific exclusion criteria for subjects with T2DM consisted of severe autonomic neuropathy, pre-proliferative and proliferative retinopathy, and renal failure [94]. The experiment was performed at the Cardiovascular Physiology Laboratory, School of Sports Science, University of Verona. We followed the recommendations regarding the management of the participants and laboratory presented in the study by Otto et al [45]. Complete silence was present in the laboratory throughout the tests. The laboratory temperature was set to 25° C. Participants were instructed not to consume caffeinated foods or drinks for 24 hours, as well as not to smoke for 8 hours, prior to testing. Participants were recommended to have at least 8 hours of night sleep. Participants visited our laboratory three times (preliminary visit; morning measures; evening measures). Experiments were performed at 6 am and 9 pm as previously done by Otto et al [45]. For each group, 50% of the subjects performed the second and third sessions on the same day, while the other subjects performed the second and third sessions in the evening and the following morning, respectively. The sample size was calculated according to the primary endpoints of our study, which were the circadian and sex differences in cf-PWV within each group. The information on changes in systolic BP, diastolic BP, and HR have a secondary role within our study and play an exploratory role. The number of subjects was initially calculated through an a priori analysis of sample size (GPower 3.1.9.7; Universität Düsseldorf, Germany) using data on circadian and sex differences in cf-PWV at rest retrieved in previous investigations. The analysis was repeated after having collected data from 10 participants (5 men and 5 women) within each group to evaluate any possible adjustment of the sample size to achieve a statistical power greater than 80% at rest and during sympathetic activation. It was obtained that (H50-80: n=16 and 20; T2DM50-80: n=18 and 22; number of subjects at rest and during sympathetic activation, respectively) were needed to assess circadian differences in cf-PWV, while (H50-80: n=22 and 26; T2DM50-80: n=24 and 28; number of subjects at rest and during sympathetic activation, respectively, to be

equally divided in men and women) were needed to assess sex differences. The sample size of 30 individuals per group we recruited is greater than that suggested by statistical analysis, and close to or greater than that of similar previous investigations [47, 92, 93]. The study was approved by the Ethics Board of the University of Verona (3293CESC) and conducted in accordance with the declaration of Helsinki. Informed oral and written consent was obtained from all participants before starting any test.

Experimental protocol

In the preliminary visit, our medical team assessed whether participants met the inclusion or exclusion criteria through physical examination, cardiovascular screening, and medical history review. Moreover, subjects completed two maximal handgrip (Saehan SH5001, Germany) contractions with their left hand to assess their maximum voluntary contraction. Each contraction lasted approximately 3 seconds and was separated by 4 minutes of rest. In the second and third sessions, participants lay supine on an ambulatory bed throughout the experiment. Participants were told to stay relaxed and breathe regularly as well as not to speak throughout the experiment. Participants were suited with the electrocardiograph of the pulsed Doppler ultrasound machine (LOGIQ S7 pro, GE, Milwaukee, USA), as well as with a beat-by-beat finger BP and HR monitoring system (Finapres Medical System BV, The Netherlands) on the third medial phalanx of the right hand. After 10 minutes of supine and quiet rest, 3 BP measurements were taken using the Riva-Rocci method on the left arm and averaged to obtain systolic and diastolic BP values to calibrate the Finapres device. After further 10 minutes, the experiment started.

The protocol consisted of 5 minutes of rest followed by 5 minutes of acute sympathetic activation triggered by handgrip exercise at 30% of maximum voluntary contraction [53]. The cf-PWV, systolic and diastolic BP, and HR were measured during the last minute of each condition. The cf-PWV assessment was performed on the right side of the body and following the guidelines on user procedures previously indicated [95]. Details about the cf-PWV assessment via Doppler Ultrasound are reported in our previous paper [96]. Briefly, scanning of the carotid artery at the supraclavicular level followed by another scanning of the

common femoral artery in the groin were performed. Measures were performed in B-mode with a pulsed Doppler Ultrasound with a Linear Array (6.6 MHZ) probe synchronized with ECG. The pulse transit time calculation was performed offline using the software installed within our Ultrasound scanner. The software required to manually place a first cursor at the R peak of the ECG signal and a second cursor at the foot of the Doppler flow to return the time elapsed between the two points. The foot of the Doppler flow wave identifies the point where the steep rise of the waveform starts as previously shown by Calabria et al [97]. The R-to-flow wave times at the carotid and femoral arteries were calculated on 15 consecutive cardiac cycles and then averaged to obtain the mean carotid and femoral pulse transit times, respectively. The carotid-femoral pulse transit time was then calculated as the absolute value of the difference between the mean carotid and femoral pulse transit times. The pulse transit distance was calculated as 0.8 times the length from the common carotid artery to the common femoral artery at the groin [95]. Finally, the cf-PWV was calculated as pulse transit distance divided by carotid-femoral pulse transit time.

Statistics

The effect of T2DM, day time, and interaction on cf-PWV, systolic BP, diastolic BP, and HR at rest was determined by comparing the data collected in H50-80 vs T2DM50-80 in the morning vs evening via 2-way repeated measures ANOVA with Sidak post-hoc test. The average value between morning and evening values of the previous variables at rest was then calculated for each subject to assess sex differences. The effect of T2DM, sex, and interaction on cf-PWV, systolic BP, diastolic BP, and HR at rest was determined by comparing the data collected in H50-80 vs T2DM50-80 in men vs women via 2-way ANOVA with Sidak post-hoc test. Rest and sympathetic activation were considered as two independent conditions. Thus, statistical analyses were repeated with the data collected during sympathetic activation. The analysis of covariance required to assess the effects of systolic BP, diastolic BP, and HR on cf-PWV in the morning compared to the evening, as well as in men compared to women, was performed with MATLAB (MathWorks, USA). Significance was set at $p<0.05$. GraphPad Prism 8 (GraphPad Software, San Diego, United States) was used for statistical analysis and graphs.

RESULTS

Characteristics of the subjects

Table 1 shows the characteristics of the subjects. The mean age in H50-80 was similar to that in T2DM50-80. Body weights and BMIs were similar in H50-80 compared to T2DM50-80. The mean duration of T2DM from diagnosis in the T2DM50-80 group was 6.2±4.8 years.

Circadian variations

As reported in Figure 1 and Table 2, cf-PWV values were lower in the morning compared to the evening in both groups at rest, while they were similar during sympathetic activation. ANOVA test results revealed significant effect of day time on cf-PWV between H50-80 and T2DM50-80 at rest ($p=0.008$) but not during sympathetic activation ($p=0.22$). There was no effect of diabetes or interaction on cf-PWV in both rest and sympathetic activation conditions ($p>0.54$). Circadian differences in cf-PWV disappeared after adjusting for systolic BP, diastolic BP, and HR in both groups, both at rest and during sympathetic activation. Systolic BP values at rest were higher in the morning compared to the evening in H50-30 and similar in the other group, while there were no circadian differences in both groups during sympathetic activation. Diastolic BP was higher in the morning compared to the evening in H50-80 and T2DM50-80 at rest, while no circadian differences were observed during sympathetic activation. HR was lower in the morning compared to the evening in H50-80 and T2DM50-80, while it was similar in T2DM50-80 and lower in H50-80 during sympathetic activation.

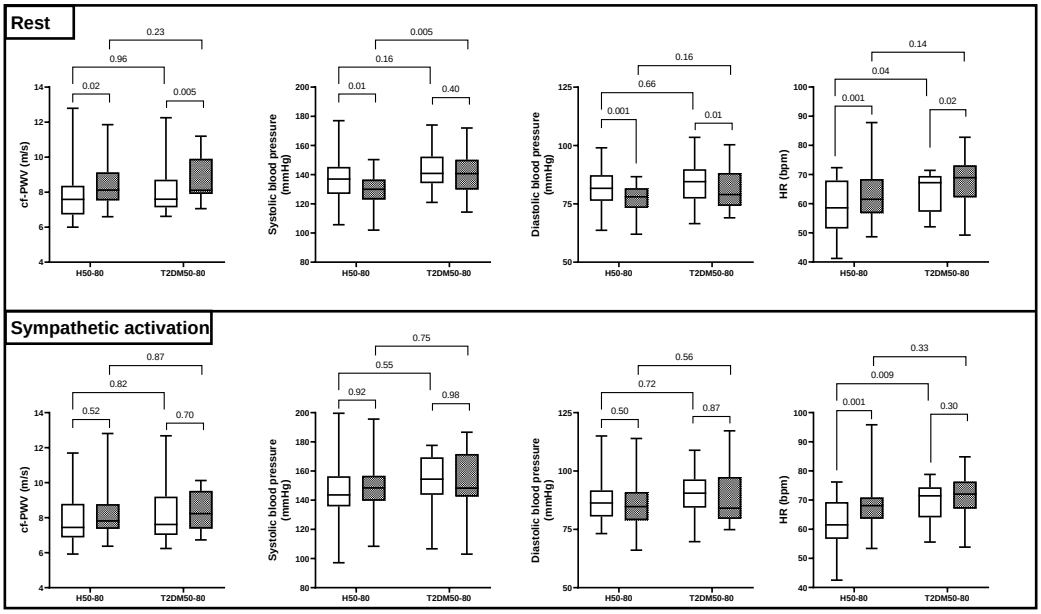

Figure 1. Circadian variations (morning: empty bars; evening: filled bars) of cf-PWV, systolic AP, diastolic AP, and HR across the 3 groups at rest and during sympathetic activation (mean, Q1/4, Q3/4, and minimum and maximum values).

Cf-PWV (m/s)		**Morning**	**Evening**	**p-value**	**Adjusted p-value**
Rest	H50-80	7.9(1.6)	8.5(1.3)	p=0.02	p>0.28
	T2DM50-80	8.0(1.4)	8.6(1.2)	p=0.005	p>0.19
Sympathetic activation	H50-80	8.2(1.4)	8.7(1.4)	p=0.52	p>0.46
	T2DM50-80	8.4(1.5)	8.7(1.1)	p=0.70	p>0.42

Table 2. Circadian changes in cf-PWV at rest and during sympathetic activation within each group. Data are reported as mean(SD). The p-value was adjusted for systolic BP, diastolic BP, and HR.

Sex differences

As reported in Figure 2 and Table 3, at rest, cf-PWV values were higher in women compared to men in both groups. During sympathetic activation, the cf-PWV was similar between sexes in H50-80 and higher in women compared to men in T2DM50-80. ANOVA test results revealed significant effect of sex on cf-PWV between H50-80 and T2DM50-80 both at rest and during sympathetic activation ($p<0.003$). There was no effect of diabetes on cf-PWV in both rest and sympathetic activation conditions ($p>0.35$). However, there was interaction during sympathetic activation ($p=0.03$) but not at rest ($p=0.85$). Sex differences in cf-PWV did not change after adjusting for systolic BP, diastolic BP, and HR in both groups, both at rest and during sympathetic activation. Systolic BP, Diastolic BP and HR values were similar in the morning compared to the evening in H50-80 and T2DM50-80 at rest and during sympathetic activation.

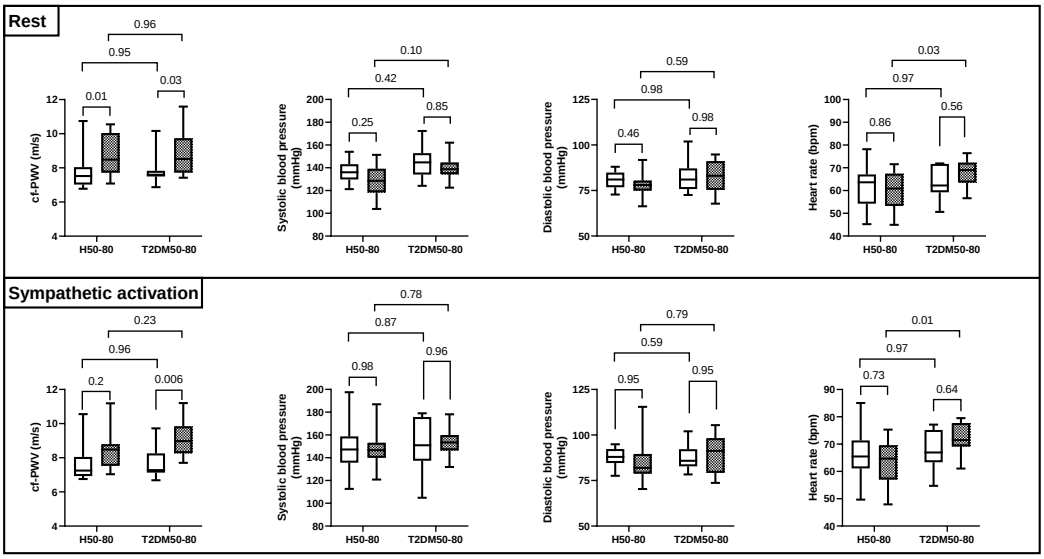

Figure 2. Sex differences (men: empty bars; women: filled bars) in cf-PWV, systolic AP, diastolic AP, and HR across the 3 groups at rest and during sympathetic activation (mean, Q1/4, Q3/4, and minimum and maximum values).

Cf-PWV (m/s)		Men	Women	p-value	Adjusted p-value
Rest	H50-80	7.7(1.0)	8.8(1.2)	p=0.01	p<0.03
	T2DM50-80	7.8(0.9)	8.9(1.4)	p=0.03	p<0.04
Sympathetic activation	H50-80	8.0(1.1)	9.0(1.1)	p=0.20	p>0.12
	T2DM50-80	7.9(1.4)	9.2(1.2)	p=0.006	p<0.01

Table 3. Sex differences in cf-PWV at rest and during sympathetic activation within each group. Data are reported as mean(SD). The p-value was adjusted for systolic BP, diastolic BP, and HR.

DISCUSSION

We investigated circadian variations and sex differences in aortic stiffness assessed via cf-PWV in old healthy individuals and old individuals with T2DM. Measures were performed in the early morning and the evening, at specific times when endothelial function, a compelling index of coronary artery disease and cardiovascular risk, is significantly different [45]. Data were collected at rest as well as during sympathetic activation triggered by a standardized external stressor. Specifically, the static handgrip exercise we used to activate the sympathetic nervous system has been shown to reliably increases muscle sympathetic nerve activity [98] and peripheral vasoconstriction [53] and used in previous studies to evaluate the role of the sympathetic nervous system in the regulation of aortic stiffness [99, 100]. Augmented aortic stiffness is an independent risk factor for cardiovascular events [90, 91]. Chronically, augmented aortic stiffness can induce deleterious remodeling of the heart and vessels as a consequence of the greater cardiac afterload, leading to conditions such as heart failure and end-organ damage [90, 91, 101]. Increases in sympathetic outflow resulting from aging and disease can further increase aortic stiffness and contribute to deleterious cardiovascular effects [101]. Interestingly, increases in sympathetic outflow have been associated with cardiac compensatory mechanisms in the presence of impaired cardiac

hemodynamics due to cardiovascular disease [101]. The focused study of the effects of the sympathetic nervous system on the heart has allowed to identify relevant indices of myocardial dysfunction that may provide prognostic implications in cardiovascular disease [101].

Circadian variations

Contrary to our working hypothesis, at rest, the cf-PWV was lower in the morning compared to the evening in both groups. ANOVA test revealed significant effect of day time on cf-PWV between H50-80 and T2DM50-80 at rest, but no effect of diabetes or interaction. A previous study showed that aortic stiffness is generally lower at night (mean value from 6.30pm to 6am) than during the day (mean value from 6.30am to 6pm) in healthy middle-aged subjects of both sexes [93]. When specific times of the day were chosen, however, another study showed that circadian variations in cf-PWV are similar in healthy young individuals and healthy elderly, at 9am compared to 5pm [92]. A study performing circadian comparisons at specific times when the endothelial function is blunted, as evinced by impaired brachial artery flow-mediated vasodilation in the morning, showed that cf-PWV is lower in old individuals with hypertension at 7am compared to 9am [47]. To the best of our knowledge, no previous studies have performed such investigations in healthy older individuals and older people with T2DM. Overall, the lower values of cf-PWV in the morning compared to the evening we found within these groups at rest agree with most previous studies. Hence, aortic stiffness appears not to be increased in the morning at specific times while endothelial function has been suggested to be blunted. Moreover, lower resting cf-PWV in the morning is present regardless of the presence of T2DM. At the present time, no study has assessed circadian variations in cf-PWV during sympathetic activation. ANOVA test revealed no effect of day time, diabetes or interaction on cf-PWV between H50-80 and T2DM50-80. The circadian variations in cf-PWV observed at rest disappeared in H50-80 and T2DM50-80, in which cf-PWV values became similar in the morning compared to the evening. Thus, aortic stiffness appears not to be increased in the morning compared to the evening even in the presence of sympathetic activation in both groups. The cf-PWV is dependent by BP and HR

[102]. Thus, normalization to BP and HR were performed to identify changes in arterial stiffness independently of confounding factors. Interestingly, circadian variations in cf-PWV disappeared after adjusting for BP and HR in both groups, suggesting that circadian changes in aortic stiffness may be largely explained by variations in these variables between morning and evening. Furthermore, when present, circadian changes in cf-PWV were small (<0.6 m/s), therefore of little concern from a clinical point of view. Similarly, the mean value of cf-PWV reached during sympathetic activation was only slightly increased compared to that at rest (<0.4 m/s). Circadian variations in BP and HR were present in H50-80. BP was higher and HR was lower in the morning compared to the evening in H50-80. Overall, the T2DM50-80 group showed similar circadian differences compared to those observed within the H50-80 group, except for a lack of circadian change in systolic BP.

Sex differences

At rest, our cf-PWV data suggest that aortic stiffness is higher in women compared to men in older individuals without and with T2DM. ANOVA test revealed significant effect of sex on cf-PWV between H50-80 and T2DM50-80 at rest, but no effect of diabetes or interaction. Our data are consistent with previous literature suggesting that aortic stiffness be lower in young women compared to young men after puberty, and that women experience a more rapid increase in artery stiffening with aging [103]. Among the causes, a key role for oestrogen in the aging-associated increases in aortic stiffening in women has been suggested [103]. Higher cf-PWV values in women with T2DM compared to men with T2DM also agree with previous studies. Indeed, increases in cf-PWV have been suggested to take place mainly in women with T2DM than men with T2DM [104]. During sympathetic activation, cf-PWV values became similar between sexes in H50-80 and persisted higher in women compared to men in T2DM50-80. ANOVA test revealed no effect of diabetes on cf-PWV between H50-80 and T2DM50-80 during sympathetic activation, but significant effect of sex and interaction. Overall, these findings suggest that aortic stiffness is higher at rest and similar under stress conditions in women compared to men in old healthy individuals, and higher in

women in both rest and stress conditions in old individuals with T2DM. Sex differences in cf-PWV persisted after adjusting for BP and HR, suggesting that a different aortic stiffness between sexes is independent by the diverse BP and HR values in men compared to women. Other factors could be responsible for different aortic stiffness between the sexes, including differences in the mechanical proprieties of the vessel, or a different sympathetic neurovascular transduction [86, 89]. Systolic BP was similar between sexes in H50-80 and T2DM50-80. Although women show lower BP values compared to men at a young age, women display a steeper increase in BP than men, which starts from the third decade and continues through the life course, even if corrected for multiple cardiovascular disease risk factors [105]. This may nullify sex differences in BP in adulthood that are otherwise present at a young age.

CONCLUSION

Cf-PWV values were lower in the morning compared to the evening within all groups at rest, while they were similar in H50-80 and T2DM50-80 during sympathetic activation. Hence, aortic stiffness appears not to be increased in the morning compared to the evening at specific times, when the endothelial function has been suggested to be blunted, regardless of the presence of a stressful condition. At rest, cf-PWV values were higher in women compared to men. During sympathetic activation, the cf-PWV was similar between sexes in H50-80 and still higher in women compared to men in T2DM50-80. Thus, older women have greater aortic stiffness compared to older men at rest, regardless of T2DM. While healthy older women show similar aortic stiffness values compared to their male counterparts during acute stress, older women with T2DM may have greater aortic stiffness compared to men with T2DM.

CHAPTER 3: CIRCADIAN VARIATIONS IN SYMPATHETIC VASOCONSTRICTION IN OLDER ADULTS WITH AND WITHOUT TYPE 2 DIABETES.

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Keywords: Aging; Diabetes mellitus; Cardiovascular disease; Risk factors.

ABSTRACT

An impact of the sympathetic nervous system in the higher rate of cardiovascular events in the early morning compared to the evening has been claimed. Augmented sympathetic vasoconstriction increases cardiovascular risk by augmenting pulse pressure and cardiac afterload. Type 2 diabetes (T2DM) further increases sympathetic neurovascular transduction and cardiovascular risk. We assessed whether peripheral vasoconstriction triggered by a standardized sympathetic stressor is augmented at 6am vs 9pm in adults between 50-80 years with type 2 diabetes (T2DM50-80) vs healthy ones (H50-80). Mean values of sympathetic vasoconstrictor responsiveness (SVR), vascular conductance (VC), brachial artery blood flow, and mean arterial pressure were measured on the contralateral forearm over two 5-minute bouts of rest and handgrip-mediated sympathetic stimulation, respectively. Although baseline VC values were lower ($p<0.01$) in the morning vs evening in both groups, SVR values in response to sympathoexcitation were similar in H50-80 (-0.43±12.44 vs -2.57±11.63 %, $p=0.73$) and T2DM50-80 (+6.64±10.67 vs +5.21±7.64 %, $p=0.90$), but higher ($p<0.01$) in T2DM50-80 vs H50-80 at both day hours. Individuals with T2DM reported positive SVR values and VC change-scores, while healthy individuals reported statistically different ($p<0.02$) negative SVR values and VC change-scores. Peripheral vasoconstrictor triggered by a standardized sympathetic stressor is similar between morning and evening, regardless of T2DM and different baseline VC values. However, peripheral vasoconstriction responsiveness is blunted in individuals with T2DM as handgrip-mediated sympathoexcitation induces vasodilation in the contralateral forearm in adults with T2DM and vasoconstriction in healthy age-matched controls, highlighting a neurovascular response altered by T2DM.

INTRODUCTION

Cardiovascular events strike more frequently in the morning compared to the evening [41, 64, 106]. A higher risk of stroke, heart attack, and cardiac death has been reported between 6am and 12am [41, 64, 106]. Among the contributors, a role for the sympathetic nervous system has been argued [42, 64, 107]. Previous findings support the notion of greater sympathetic-mediated cardiovascular effects in the morning. Arterial pressure and forearm vascular resistance are augmented in the early morning [44]. Rapid eye movement-related augmented sympathetic activation is particularly evident in the early morning [45, 46]. Endothelial vasodilation function, which can be restrained by the sympathetic outflow [19, 86], is blunted in the early morning [45, 47]. However, whether peripheral vasoconstriction triggered by a sympathetic stressor is augmented in the morning vs evening has not yet been defined. Several stressors can acutely activate the sympathetic nervous system, including sustained muscle contractions, mental stress, and emotions [19]. Some of these can easily show up in daily life. Augmented peripheral vasoconstriction in response to sympathetic stressors can increase pulse pressure and arterial stiffness, and thereby the risk for cardiovascular events by augmenting the afterload, work, and oxygen demand of the heart and the formation of vascular lesions [44, 53]. Any augmented peripheral vasoconstriction responsiveness in the morning could thus represent an important aspect involved in the higher morning rate of cardiovascular events.

The rate of cardiovascular events increases in the presence of type 2 diabetes (T2DM) [40, 55, 56]. T2DM leads to deleterious alterations in the autonomic pathways controlling circulation, arterial pressure, and feedback baroreceptor system sensitivity [64]. Neuropathy and symptoms of postural hypotension in individuals with T2DM suggest that the effects of T2DM affect the sympathetic branch of the autonomic nervous system [59, 63]. T2DM augments sympathetic dominance [59, 63, 64]. Interestingly, subjects with T2DM exhibit augmented sympathetic transduction to blood pressure compared to healthy controls [65], which is strictly connected to peripheral vasoconstriction in response to sympathetic stressors. However, whether subjects with T2DM exhibit enhanced peripheral vasoconstriction responsiveness compared to healthy age-matched

individuals in response to standardized sympathetic stressors has never been investigated.

Sympathetic vasoconstrictor responsiveness (SVR) is a conventional variable for quantifying the extent of peripheral vasoconstriction in response to a sympathetic stressor and is defined as the percent change in vascular conductance (VC) from the preceding baseline value [7, 108]. SVR reflects the change in resistance vessel radius over different values of baseline VC, including changes at the microvascular level [7, 108]. We assessed whether peripheral vasoconstriction triggered by a standardized sympathetic stressor, measured via SVR, is enhanced in the morning compared to the evening in healthy older adults and age-matched individuals with T2DM, hypothesizing that vasoconstriction responsiveness is augmented in the morning in both groups. We also assessed whether peripheral vasoconstriction differs between healthy older adults and age-matched individuals with T2DM, hypothesizing that vasoconstriction responsiveness is enhanced in individuals with T2DM.

MATERIAL AND METHODS

60 participants were recruited and divided into the following two sex-balanced groups: 30 healthy individuals from 50 to 80 years old (H50-80) and 30 individuals with T2DM from 50 to 80 years old (T2DM50-80). All participants met common inclusion (>18 years old) and exclusion criteria (chronic hypertension, atrial fibrillation, cardiac valve disease, pacemaker-dependent, use of beta-blockers and ACE inhibitors, pregnancy or presumed pregnancy) [94]. Healthy subjects had to have fasting blood glucose lower than 100 mg/dL. Subjects with T2DM had to have been diagnosed with T2DM for at least 1 year, as well as to be free of severe autonomic neuropathy, pre-proliferative and proliferative retinopathy, and renal failure [94]. Subjects were instructed to avoid any caffeinated foods or drinks for 24 hours and to have at least 8 hours of night sleep before the experiments. Participants reported to the laboratory three times (preliminary visit; morning measures; evening measures). Tests were performed at 6am and 9pm, when the endothelial function is statistically different [45]. The second and third sessions

were on the same day for 50% of the subjects of each group, whereas on the evening and the following morning for the other subjects.

Experimental protocol

In the preliminary visit, the maximum voluntary contraction of the left forearm was measured. Participants performed two maximal handgrip (Saehan SH5001, Germany) contractions with their left hand, each of approximately 3 seconds and separated by 4 minutes of rest. In the second and third sessions, participants lay supine on an ambulatory bed throughout the experiment. The laboratory was noiseless and temperature-controlled (25° C). The subjects' right arm was extended on a support for ultrasound measurements. Participants were instructed to stay relaxed, breathe regularly, and not to speak throughout the test. Participants were suited with an automatic arterial pressure monitor (Tango+, SunTech Medical, Morrisville, NC; USA) at the heart level on their left arm, with the 3-lead electrocardiograph (ECG) of the Ultrasound Device (LOGIQ S7 pro, GE, Milwaukee, USA), and with a beat-by-beat finger arterial pressure monitoring system (Portapres; Finapres Medical System BV, The Netherlands) on the third medial phalanx of the right hand. Portapres measured the mean arterial pressure (MAP) and was calibrated on the brachial arterial pressure. Subsequently, the right brachial artery started to be scanned via pulsed Doppler ultrasonography to concurrently measure mean blood velocity and brachial artery diameter. The probe was located above the antecubital fossa. The probe position was marked for measuring the same artery section in the morning and evening. The ultrasound gate was set to scan the full artery width. The sample volume was aligned and set accordingly to the vessel dimension. Ultrasound data were collected by an expert sonographer with >500 hours of experience. Once the experimental setup was ready, after an additional 15 minutes of rest, the test started as follow. The procedure consisted of 5 minutes of rest followed by 5 minutes of acute sympathetic activation induced by handgrip exercise at 30% of the maximum voluntary contraction [53, 64, 98, 109, 110]. The handgrip exercise was performed with the

left hand. Ultrasound and MAP measures were collected on the contralateral arm throughout the 10 minutes of testing.

Data analysis

Brachial artery diameter and mean blood velocity were automatically detected via software (Medical Imaging Applications LLC, USA) from the ultrasound video clips as previously done [86]. Brachial artery diameter was detected at the onset of each R-wave of the ECG. MAP data were exported from the proprietary software of Portapres (BeatScope 1.1; Finapres Medical System BV, The Netherlands). Mean values of brachial artery diameter, mean blood velocity, and MAP were calculated within the 5 minutes of rest and the 5 minutes of sympathetic activation, as well as at each minute of sympathetic stimulation. The mean brachial artery blood flow (BF) values at rest and during sympathetic activation were calculated as mean blood velocity (cm*s−1)*π*(mean brachial artery radius)^2*60 (ml*min−1). Mean VC values at rest and during sympathetic activation were calculated as the average value of brachial BF divided by the average value of MAP. SVR was calculated as the percent change in VC during sympathetic activation from the preceding value at rest [7, 108]. Absolute change-scores of VC, BF, and MAP from rest to sympathetic activation conditions were calculated.

Statistics

To compare morning vs evening measures in H50-80 vs T2DM50-80, SVR data were assessed via two-way repeated measures ANOVA with Sidak post-hoc test, while change-scores of VC, BF, and MAP were assessed via repeated measure ANCOVA with baseline values used as covariates and Tukey post-hoc test. Moreover, to identify differences in SVR and VC change-scores over the stimulation time in H50-80 vs T2DM50-80, regardless of circadian variations, morning and evening values of SVR and VC change-scores were averaged and assessed via two-way repeated measures ANOVA with Sidak post-hoc test. The

average value between morning and evening values of SVR and change-scores in VC, BF, and MAP was calculated and compared between men vs women and H50-80 and T2DM50-80 to assess the effect of sex, T2DM, and their interaction. Statistical significance was set at $p<0.05$. Due to the lack of similar previous studies, the statistical power was assessed a-posteriori to ensure that changes in all variables between morning and evening, as well as between H50-80 and T2DM50-80, reached a statistical power >80%. The true differences in SVR, VC, BF, and MAP from baseline to sympathoexcitation and between groups were used to determine the effect sizes. Then, statistical power was assessed via software (GPower 3.1.9.7; Universität Düsseldorf, Germany) by setting an F test (ANOVA, repeated measures, within-between interaction), effect sizes, and level of significance $\alpha=0.05$. Our data suggested that a statistical power >80% in all variables could have been achieved with 24 healthy individuals vs 24 individuals with T2DM. Results are reported as mean±SD. GraphPad Prism 8 (GraphPad Software, San Diego, United States) and MATLAB (MathWorks, USA) were used for statistical analysis and graphs.

RESULTS

The mean age in H50-80 was similar to that in T2DM50-80 (66.4±7.5 vs 66.9±7.6 years, p=0.96). Body weights (74.3±13.0 vs 82.8±18.6 Kg, p=0.10), heights (1.67±0.09 vs 1.69±0.12 m, p=0.81), and BMIs (26.8±4.1 vs 29.0±5.9 kg/m^2, p=0.16) were similar between groups.

ANOVA test results and F-values are reported in the supplementary file. Baseline VC values were lower in the morning vs evening in both H50-80 (1.12±0.49 vs 1.45±0.57 mL/min/mmHg, p=0.001) and T2DM50-80 (1.10±0.39 vs 1.36±0.62 mL/min/mmHg, p=0.01), but did not differ (p>0.80) between H50-80 and T2DM50-80 either in the morning or the evening. Baseline BF values were lower in the morning vs evening in H50-80 (109.8±45.7 vs 137.5±52.7 mL/min, p=0.008) and T2DM50-80 (115.5±46.3 vs 137.9±63.6 mL/min, p=0.03), but did not differ between groups at both day hours (p>0.90). Baseline MAP values were higher in the morning vs evening in H50-80 (99.5±9.3 vs 94.8±5.2 mmHg, p=0.004) but not in T2DM50-80 (103.7±9.1 vs 101.2±10.1 mmHg, p=0.28).

Baseline MAP differed between groups in the evening (p=0.02) but not in the morning (p=0.22).

As shown in Figure 1, SVR was similar in the morning vs evening in H50-80 (-0.43±12.44 vs -2.57±11.63 %, p=0.73) and T2DM50-80 (+6.64±10.67 vs +5.21±7.64 %, p=0.90), but higher (p<0.01) in T2DM50-80 vs H50-80 at both day hours. The VC change from rest to sympathoexcitation was similar in the morning vs evening in H50-80 (-0.03±0.16 vs -0.03±0.18 mL/min/mmHg, p=0.96) and T2DM50-80 (+0.07±0.13 vs +0.06±0.12 mL/min/mmHg, p=0.93). However, the VC change was higher (p<0.02) in T2DM50-80 vs H50-80 at both day hours. The BF change was similar in the morning vs evening in H50-80 (+6.4±10.9 vs +11.2±17.6 mL/min, p=0.46) and T2DM50-80 (+17.9±16.0 vs +19.0±14.6 mL/min, p=0.96), but higher in T2DM50-80 vs H50-80 in the morning (p=0.01) but not in the evening (p=0.20). The MAP change was lower in the morning vs evening in H50-80 (+9.1±5.8 vs +12.1±6.7 mmHg, p=0.01) but similar in T2DM50-80 (+9.0±5.4 vs +9.3±5.6 mmHg, p=0.90). However, the MAP change was similar between H50-80 and T2DM50-80 in the morning (p=0.90) and in the evening (p=0.25).

As reported in Figure 2, regardless of circadian variations, individuals with T2DM report positive values of SVR and absolute change-scores of VC, while healthy individuals report statistically different negative values of SVR (p<0.01) and absolute change-scores of VC (p<0.03) at each minute of stimulation.

When sex differences were assessed, regardless of circadian variations, ANOVA results revealed a significant effect of T2DM in SVR and change-scores of VC and BF (p<0.004), but not on MAP (p=0.37). However, there was no effect of sex or interaction with T2DM in any variable (p>0.47).

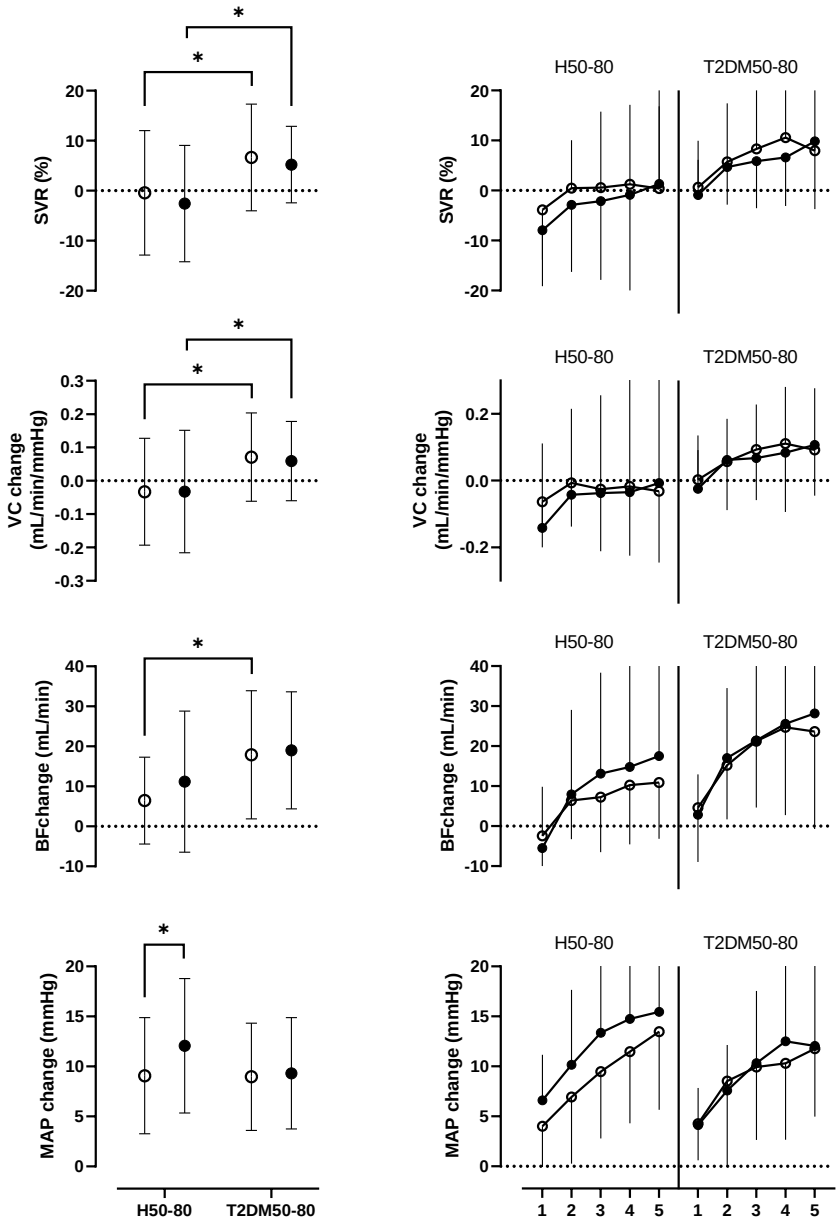

Figure 1. The left panel shows sympathetic vasoconstrictor responsiveness (SVR) and absolute change-scores from rest to sympathetic activation conditions in vascular conductance (VC), blood flow (BF), and mean arterial pressure (MAP) adjusted for baseline values as covariate, in the morning (white dots) vs evening (black dots), in healthy older adults (H50-80) vs older adults with type 2 diabetes (T2DM50-80). The right panel shows the changes of the previous variables over the 5-minute stimulation time.

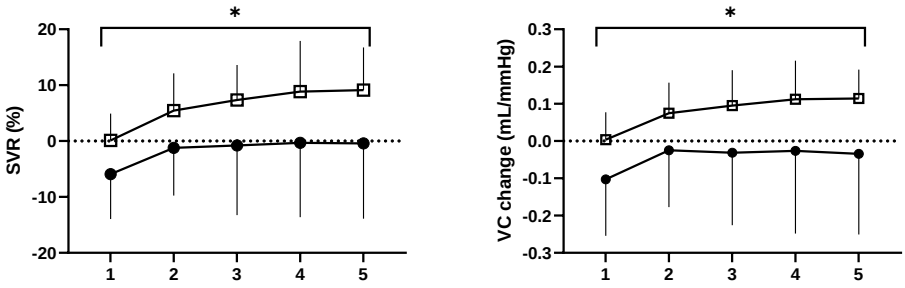

Figure 2. SVR and absolute change-scores of VC over the 5 minutes of stimulation in healthy older adults (black dots) vs older adults with type 2 diabetes (white squares). The graphs report the average between morning and evening SVR and VC values.

DISCUSSION

We assessed whether peripheral vasoconstriction triggered by a standardized sympathetic stressor, measured via SVR, is augmented in the morning than in the evening in healthy older adults and age-matched individuals with T2DM. Moreover, we assessed whether peripheral vasoconstriction responsiveness is augmented in individuals with T2DM than in healthy ones as the former exhibit augmented sympathetic transduction to blood pressure compared to the latter [65]. Tests were performed at 6am and 9pm, when the endothelial function, a reliable index of cardiovascular risk, is statistically different and blunted in the morning [45, 47]. Sympathetic vasoconstrictor responsiveness (SVR) is a conventional variable for quantify the extent of peripheral vasoconstriction in response to sympathetic stressors, providing the percent change in VC from rest to sympathetic activation conditions and information that is normalized over different baseline values of VC [7, 108]. The first finding was that, although baseline VC values were lower in the morning than in the evening, the SVR in response to sympathoexcitation was similar between morning and evening in both groups. The second but most unexpected finding is that, regardless of the time of day, individuals with T2DM reported positive SVR values and change-scores of VC in response to sympathetic stimulation, indicating an increase in VC (vasodilation). In contrast, healthy

individuals reported statistically different negative SVR values and change-scores of VC, indicating a reduction in VC (vasoconstriction). Thus, the peripheral vasoconstriction triggered by a standardized sympathetic stressor is blunted in individuals with T2DM than in healthy ones as the stressor led to vasodilation on the contralateral forearm in individuals with T2DM and vasoconstriction in healthy age-matched subjects over the 5-minute stimulation.

Circadian variations in peripheral vasoconstriction responsiveness

Whether stress factors capable of activating the sympathetic nervous system could trigger cardiovascular events and whether these may lead to greater cardiovascular risk in the morning vs evening have often been questioned [42, 64, 107]. Any augmented peripheral vasoconstriction in response to sympathetic stressors in the morning may be an important aspect involved in the higher morning rate of cardiovascular events by increasing the pulse pressure and arterial stiffness, and thereby the cardiac afterload and the formation of vascular lesions [44, 53]. In agreement with previous studies, baseline VC values were lower in the early morning vs evening in both groups [44]. This finding is consistent with greater baseline sympathetic outflow and blunted endothelial function previously reported in the early morning vs evening [42, 44–47]. However, contrary to our working hypothesis, SVR was similar in the morning vs evening in both groups. Furthermore, the change-scores from rest to sympathetic activation in VC, even after adjusting for differences in baseline values, were similar between morning and evening in both groups. Thus, these findings suggest that although baseline VC values could be lower in the early morning, the peripheral vasoconstriction triggered by a standardized sympathetic stressor is similar between morning and evening, regardless of the presence of T2DM. These results also agree with our previous findings showing that carotid-femoral pulse wave velocity, another variable affected by the effects of peripheral vasoconstriction on blood pressure, is not augmented at 6am vs 9pm in older adults with and without T2DM, not only at rest but also during handgrip-mediated sympathoexcitation [64].

Comparison between older adults with and without T2DM

In our study, sympathetic activation was induced by handgrip exercise at 30% of the maximum voluntary contraction. This stressor induces sympathetic activation through physical exertion mediated by sustained muscle contraction, a condition that can easily occur in ordinary life both in the morning and in the evening. This stressor has been shown to reliably increase the muscle sympathetic nerve activity [98, 109, 110], as well as to increase MAP and vascular resistance of the lower limb [53, 64, 98, 110]. We have previously shown that this stressor restrains the VC of the leg within 2 minutes of stimulation [53]. Sympathetic activation induces peripheral vasoconstriction by releasing norepinephrine from sympathetic nerve endings and adrenal glands, which binds to post-synaptic α-adrenergic receptors on vascular smooth muscle to promote calcium influx [53]. However, previous studies that specifically focused on the upper limb also reported the possibility of neurogenic vasodilation on the contralateral arm during handgrip stimulation in healthy younger individuals [111, 112], although these vasodilator effects were transitory and evident at different magnitudes in the first minutes of stimulation. The mechanisms responsible for the handgrip-mediated contralateral forearm vasodilation have not been fully elucidated, but previous findings support the notion that it is sympathetic-mediated, through adrenaline release and β-adrenergic mechanisms [111, 112]. In agreement with the previous notions, our SVR data show that both vasoconstriction and vasodilation can occur at different time points over the stimulation time (Figure 1, right panel), with small differences between morning and evening. However, the overall response over the 5 minutes of stimulation (Figure 1, left panel) resulted in vasoconstriction at both day hours in our cohort of healthy older adults according to negative SVR values and VC change-scores. In contrast, handgrip-mediated sympathoexcitation resulted in overall vasodilation at both day hours in individuals with T2DM according to statistically different positive SVR values and VC change-scores. When the inter-group response to sympathoexcitation was tested (Figure 2), regardless of circadian variation, individuals with T2DM reported positive SVR values and change-scores of VC (vasodilation) that were statistically different from negative ones (vasoconstriction) of healthy individuals at each minute of stimulation. This finding

reveals that the peripheral vasoconstriction in response to handgrip-mediated sympathetic activation is blunted in individuals with T2DM than in healthy ones and highpoints a neurovascular response altered by T2DM. A possible explanation might be that vasodilation effects prevail over vasoconstriction effects during handgrip-mediated sympathoexcitation in subjects with T2DM, or vice versa in healthy individuals. Despite subjects with T2DM have been suggested to exhibit augmented sympathetic transduction to blood pressure compared to healthy controls [65], the MAP change in response to sympathetic activation did not differ between the two groups in our study. Peripheral vasodilation during sympathoexcitation might be a factor involved in the dampening of central blood pressure augmentation in individuals with T2DM. Although the sympathetic neurovascular transduction has been suggested to differ between sexes [64, 86], regardless of circadian variations, this T2DM-mediated blunting of peripheral vasoconstriction responsiveness takes place similarly in both sexes as ANOVA results revealed no effect of sex or interaction on the variables of interest in this study. Specific studies are required to elucidate the precise underlying mechanisms altered by T2DM on the peripheral vasoconstriction responsiveness. Changes in sympathetic outflow and remodeling of autonomic pathways mediated by T2DM might be involved [59, 63, 64]. Muscle sympathetic nerve activity and adrenaline release might also differ in response to standardized external stressors in individuals with T2DM compared to healthy ones.

CONCLUSION

The peripheral vasoconstriction triggered by a standardized sympathetic stressor is similar between morning and evening, regardless of the presence of T2DM and reduced baseline VC values in the morning. Moreover, the peripheral vasoconstriction responsiveness is blunted in individuals with T2DM than in healthy ones as handgrip-mediated sympathetic activation induced vasodilation on the contralateral forearm in individuals with T2DM and vasoconstriction in healthy age-matched subjects, highlighting a neurovascular response to an external stressor altered by T2DM.

CHAPTER 4: CIRCADIAN AND SEX DIFFERENCES IN POST-ISCHEMIC VASODILATION AND REACTIVE HYPEREMIA IN YOUNG INDIVIDUALS AND ELDERLY WITH AND WITHOUT TYPE 2 DIABETES.

Currently under review in Microcirculation.

Keywords: Aging; Diabetes mellitus; Cardiovascular disease; Risk factors; Sympathetic activation; Sex differences; Circadian changes.

ABSTRACT

As cardiovascular events strike more frequently in the morning compared to the evening and with different prevalence between sexes, we assessed circadian variations and sex differences in vascular conductance (VC) and blood flow (BF) regulations following a brief bout of forearm ischemia in young healthy individuals (H18-30) and elderly without (H50-80) and with type 2 diabetes (T2DM50-80). Forearm VC and BF, and mean arterial pressure at baseline and following circulatory reperfusion were measured at 6am and 9pm. In the morning compared to evening, following reperfusion, the VC and BF increments were similar in H18-30 ($p>0.71$), but lower in H50-80 ($p<0.001$) and T2DM50-80 ($p<0.01$). VC and BF following circulatory reperfusion were higher in men than women in H18-30 ($p<0.001$), but similar between sexes in the older groups ($p>0.23$). Forearm vasodilation following reperfusion is attenuated in the early morning in the elderly and such an attenuated vasodilation capacity impairs BF towards an ischemic area. Type 2 diabetes does not affect the circadian regulation of VC and BF, but that of MAP. There are sex differences in VC and BF at baseline and after circulatory reperfusion at a young age, being greater in men, which disappear with aging without being affected by diabetes.

INTRODUCTION

Cardiovascular events occur more frequently in the morning compared to the evening [41, 44, 106]. This fact has led to a growing interest in the investigation of the circadian variations of physiological variables potentially related to cardiovascular risk. The brachial artery flow-mediated vasodilation (FMD) following a period of ischemia is attenuated in the morning compared to the evening, suggesting blunted endothelial function and a greater risk of coronary artery disease in the morning [45, 47]. However, artery FMD represents the percent change in diameter of a single conduit segment [86, 113]. This variable does not provide information on the entire limb vasodilation, including changes at the microvascular level, or on the precise neurovascular control of limb blood flow (BF) [86, 113]. Conversely, the sudden increase in limb vascular conductance (VC) following circulatory reperfusion reflects vasodilation of the whole limb vascular tissues and is a key regulator of limb BF along with the mean arterial pressure (MAP) [113]. The sudden increase in BF following circulatory reperfusion, named reactive hyperemia, also provides information on cardiovascular risk [114–116]. Attenuated reactive hyperemia has been related to several risk factors, such as obesity, total/HDL cholesterol ratio, type 2 diabetes (T2DM), smoking, and dyslipidemia [114–116]. VC at baseline is lower in the morning compared to the evening, suggesting a greater constriction of the vascular tissue in the morning [44]. However, it is still unknown whether the limb vasodilation capacity following circulatory reperfusion is likewise attenuated in the morning and whether any circadian restriction of vasodilation impairs subsequent reactive hyperemia towards a downstream ischemic area.

Aging is linked to an increase in cardiovascular events [41, 44, 106]. The presence of T2DM further increases such an occurrence [72, 117]. There are also overt sex differences in the incidence of cardiovascular events [15, 86]. After menopause, women start being at a greater cardiovascular risk compared to men due to the presence of a greater number of risk factors, as well as to the lack of the protective effects of oestrogen on cardiovascular health [15]. These sex differences are more evident in individuals with T2DM [84, 118]. Women with T2DM show earlier and more deleterious pathophysiological changes in cardiovascular risk-

related variables compared to men, as well as greater cardiac risk [84, 118]. Considering the different prevalence of cardiovascular events between morning and evening, as well as the impact of sex on cardiovascular risk, this study primarily aims to assess circadian variations of VC and reactive hyperemia following circulatory reperfusion in healthy elderly and elderly with T2DM of both sexes. It is hypothesized that the VC increment following circulatory reperfusion be blunted in the morning compared to the evening in both groups, and that any restriction of vasodilation impairs BF towards the ischemic area. It is also hypothesized that any sex differences in VC and BF increments following reperfusion be more marked in individuals with T2DM compared to healthy individuals. Since the endothelial function is attenuated at 6 am compared to at 9 pm [45], evaluations will be performed according to such a timing schedule.

MATERIALS AND METHODS

30 healthy individuals from 18 to 30 years old (H18-30), 30 healthy individuals from 50 to 80 years old (H50-80), and 30 individuals with T2DM from 50 to 80 years old (T2DM50-80) were recruited for this study (Age: 23.2±3.5 vs 66.4±7.5 vs 66.9±7.6 years old; Weight: 65.6±11.2 vs 74.3±13.0 vs 82.8±18.6 Kg; Height: 1.74±0.09 vs 1.67±0.09 vs 1.69±0.12 m; BMI: 21.6±2.2 vs 26.8±4.1 vs 29.0±5.9 Kg/m^2; H18-30 vs H50-80 vs T2DM50-80 respectively). All groups were sex balanced. All participants met common inclusion (>18 years old) and exclusion criteria (chronic hypertension, pacemaker-dependent, use of beta-blockers and ACE-inhibitors, pregnancy or presumed pregnancy) [94, 119]. Healthy subjects had to report fasting blood glucose lower than 100 mg / dL. Subjects with T2DM had to have been diagnosed with T2DM for at least 1 year, as well as to be free of severe autonomic neuropathy, pre-proliferative and proliferative retinopathy, and renal failure [94]. Subjects were instructed to avoid caffeine for 24 hours prior to testing, as well as to sleep at least 8 hours the night before the experiments [120]. Tests were performed at 6 am and 9 pm, when the endothelial function is different [45], at the Cardiovascular Physiology Laboratory, School of Sports Science, University of Verona. The laboratory temperature was controlled at 25° C. Participants reported to the laboratory twice for morning and evening measures, respectively.

Within each group, 50% of the subjects performed the experiments in the morning-evening order, whereas the other 50% performed the experiments in the evening and the following morning. The primary endpoints of our study were the circadian and sex differences in VC and BF within each group. Because of the lack of specific studies, the sample size within each group was determined via software (GPower 3.1.9.7; Universität Düsseldorf, Germany) after having collected data from 10 participants in each group. Such an analysis suggested the need for (H18-30: n=14; H50-80: n=18; T2DM50-80: n=20) subjects to reach a statistical power of 80% in the assessment of circadian changes in VC and BF, while the need for (H18-30: n=18; H50-80: n=24; T2DM50-80: n=26; to be equally divided in men and women) subjects to reach a statistical power of 80% in the assessment of sex differences. The comparison of differences among groups has only an explorative role within this study. The study was approved by the Ethics Board of the University of Verona (3293CESC) and conducted following the declaration of Helsinki. Informed oral and written consent was obtained from all participants before starting any test.

Experimental protocol

Both in the morning and evening sessions, participants lay supine on an ambulatory bed throughout the test with their right arm extended on a support for ultrasound measures. Subjects were instructed to stay relaxed, breathe regularly, and not to speak throughout the experiment. Participants were suited with a beat-by-beat finger arterial pressure monitoring system (Portapres; Finapres Medical System BV, The Netherlands), properly calibrated on the brachial artery arterial pressure, on the third medial phalanx of the left hand recording the MAP. Participants were also suited with the 3-lead electrocardiograph (ECG) of the Ultrasound Device (LOGIQ S7 pro, GE, Milwaukee, USA). Subjects were suited with a pressure cuff around the right forearm and distal to the imaged artery. A rapid cuff inflator (Hokanson, Bellevue, USA) was used to inflate the cuff >50 mmHg above systolic arterial pressure and to deflate the cuff within approximately 300 ms. While subjects were recommended to stay relaxed and breathe regularly, scanning of the right brachial artery via pulsed Doppler ultrasonography was

started. Ultrasound data consisted of the concurrent measure of mean blood velocity and brachial artery diameter. The brachial artery was scanned above the antecubital fossa. The probe location was marked to evaluate the same artery section in the morning and the evening. Data were measured with a 4.4 MHz probe and a 60° angle of insonation. The ultrasound gate was regulated to scan the whole artery width. The sample volume was aligned and regulated according to vessel size. Ultrasound measures were performed by an expert sonographer with >500 hours of experience. Once these procedures were completed, the main experiment started as follow. Participants performed 15 minutes of complete rest. Participants were steady during the resting time. The forearm cuff on the right arm was thus inflated for 5 minutes and then suddenly released. Ultrasound and Portapres data were collected during a 3-minute baseline before cuff inflation, during cuff inflation, and during the 3 minutes after cuff release. Data were synchronized throughout the test by using signal markers.

Data analysis

Video analysis software (Medical Imaging Applications LLC, USA) was used to detect the brachial artery diameter and mean blood velocity from the ultrasound video clips. Brachial artery diameter was measured at the onset of each R-wave of the ECG. Arterial pressure data were exported from the proprietary software of Portapres (BeatScope 1.1; Finapres Medical System BV, The Netherlands). The mean values of brachial artery diameter, mean blood velocity, and MAP were calculated at baseline as well as during the 30, 60, and 90 seconds after cuff release. Mean values of brachial artery diameter and mean blood velocity were used to calculate the mean values of BF as follows: mean blood velocity $(cm*s-1)*\pi*r^2*60$ $(ml*min-1)$, where r is the mean brachial artery radius. Similarly, mean values of VC at baseline and at different time points after circulatory reperfusion were calculated by dividing mean values of BF by mean values of MAP recorded at baseline and during the 30, 60, and 90 seconds after cuff release.

Statistics

Data passed the normality test. Within each group, circadian variations in VC, BF, and MAP at baseline and over the 30s, 60s, and 90s after circulatory reperfusion were identified via two-way repeated-measures ANOVA and Sidak post-hoc test. The effect of T2DM on VC, BF, and MAP changes was determined via 3-way repeated measures ANOVA by comparing the data collected in H50-80 vs T2DM50-80 in the morning vs evening. The average values between morning and evening values of VC, BF, and MAP were used to assess differences between sexes. Within each group, sex differences in VC, BF, and MAP at baseline and over the 30s, 60s, and 90s after circulatory reperfusion were identified via paired two-way repeated-measures ANOVA and Sidak post-hoc test. The effect of sex on VC, BF, and MAP changes was determined via 3-way repeated measures ANOVA by comparing the data collected in H50-80 vs T2DM50-80 in men vs women. Statistical significance was set at $p<0.05$. GraphPad Prism 8 (GraphPad Software, San Diego, United States) was used to perform statistical analysis and graphs. Results are reported as mean ± standard deviations.

RESULTS

As shown in Figure 1, baseline VC was higher in H18-30 compared to the other groups ($p<0.03$) and similar between H50-80 and T2DM50-80 ($p=0.87$). However, no differences in VC were noted after circulatory reperfusion among groups ($p>0.13$). Baseline BF did not differ among groups ($p>0.84$), but it was greater in H50-80 compared to H18-30 ($p<0.01$) after cuff release. MAP was greater in H50-80 compared to H18-30 ($p<0.01$), as well as in T2DM50-80 compared to H50-80 ($p<0.01$), both at baseline and after cuff release.

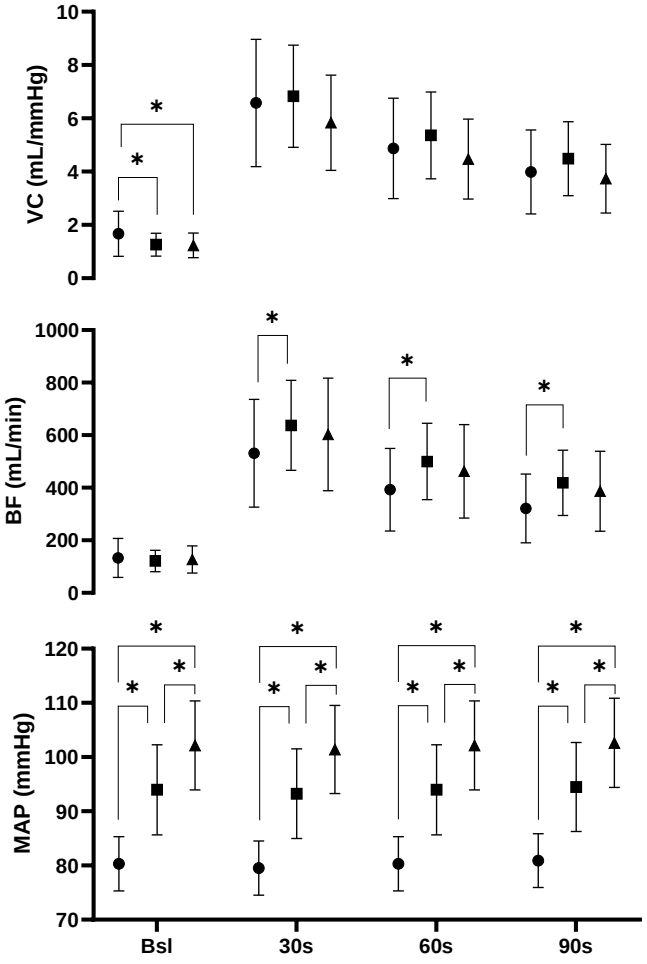

Figure 1. Differences in vascular conductance (VC), blood flow (BF), and mean arterial pressure (MAP) at baseline and after circulatory reperfusion across the three groups (●H18-30; ■H50-80; ▲T2DM50-80).

Circadian variations

As shown in Figure 2, values of VC, BF, and MAP at baseline and after circulatory reperfusion were similar in the morning compared to the evening in H18-30 ($p>0.71$). Values of VC and BF at baseline and after circulatory reperfusion were lower in the morning compared to the evening in H50-80 ($p<0.001$) and T2DM50-80 ($p<0.01$). Values of MAP were similar in the morning compared to the evening in H50-80 ($p>0.53$) but higher in T2DM50-80 ($p<0.01$). ANOVA test

revealed significant effect (p=0.005) of day time on VC and BF changes in H50-80 and T2DM50-80, but no circadian effect on MAP (p>0.50). There were no effects of T2DM or interaction on VC and BF changes (p>0.33), while there were significant effects on MAP (p<0.01).

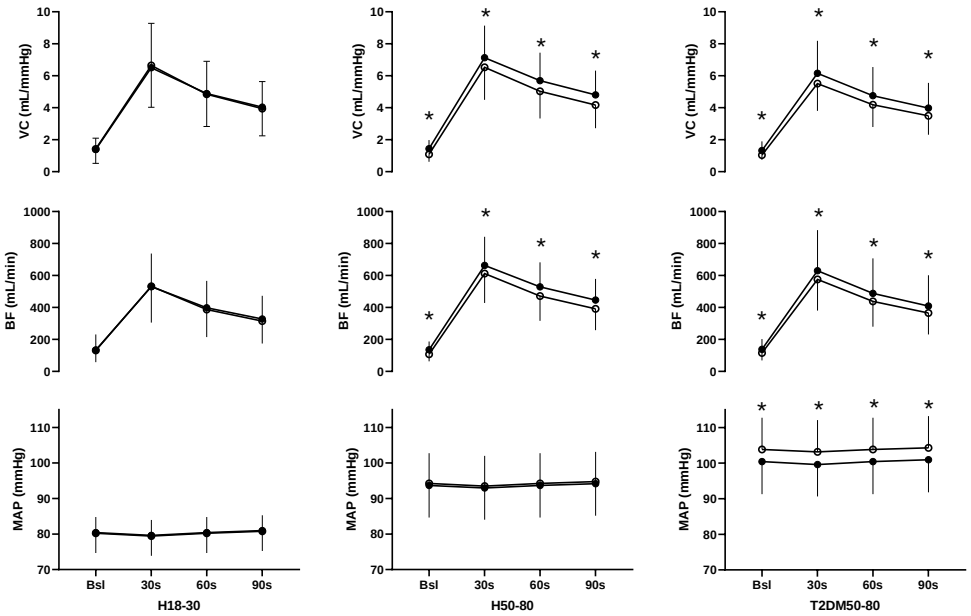

Figure 2. Changes in vascular conductance (VC), blood flow (BF), and mean arterial pressure (MAP) at baseline (Bsl) and 30s, 60s, 90s after circulatory reperfusion in the morning (○) compared to the evening (●) within each group.

Sex differences

As shown in Figure 3, values of VC, BF, and MAP at baseline and after circulatory reperfusion were higher in men compared to women in H18-30 (p<0.001), while no sex differences in such variables were noticed in the older groups (p>0.23). ANOVA test revealed no effect of sex on VC, BF, and MAP in H50-80 and T2DM50-80 (p>0.29), but there was significant effect of T2DM on MAP (p<0.03) but not on VC and BF (p>0.40).

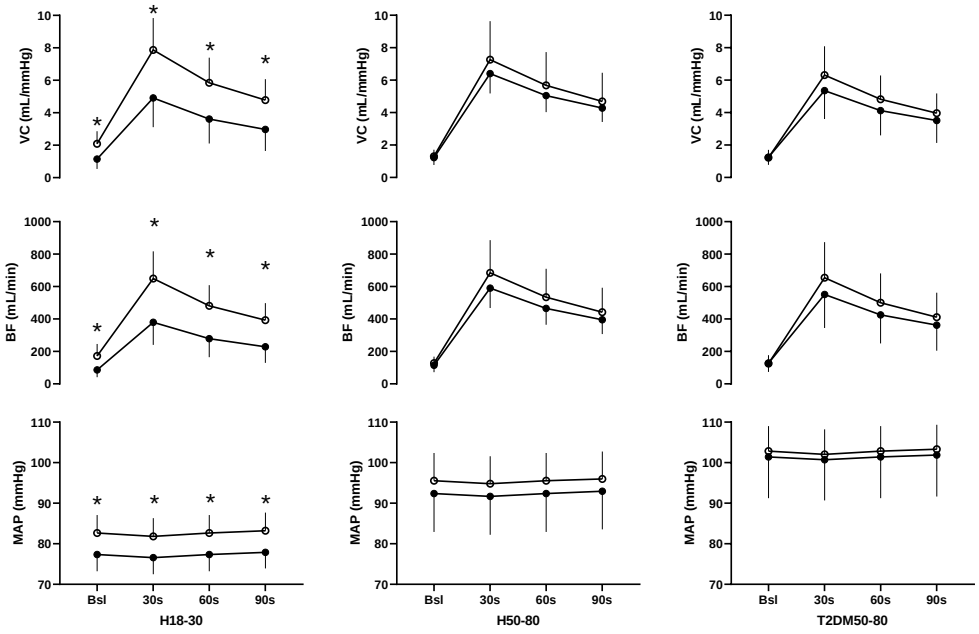

Figure 3. Changes in vascular conductance (VC), blood flow (BF), and mean arterial pressure (MAP) at baseline (Bsl) and 30s, 60s, 90s after circulatory reperfusion in men (○) compared to women (●) within each group.

DISCUSSION

We investigated circadian and sex differences in VC and BF following circulatory reperfusion in young individuals and elderly with and without type 2 diabetes. Measures were performed at 6 am compared to at 9 pm, when the endothelial function is attenuated in the morning compared to the evening [45]. VC at baseline was higher in young individuals compared to the older ones, consistent with the notion of an increase in vascular peripheral resistance with aging. Augmented sympathetic outflow along with diminished nitric oxide bioavailability have been suggested to account for the increase in peripheral resistance with aging [49–53]. However, the increase in VC after circulatory reperfusion was similar among groups. In this regard, the dynamic regulation of vascular conductance is based on the interaction between metabolic vasodilation and sympathetic vasoconstriction [7]. The sudden release of vasodilator agents following circulatory reperfusion might effectively counteract any sympathetic-mediated restriction in

VC within any group [86]. MAP at baseline and after circulatory reperfusion was greater in healthy elderly compared to healthy young individuals, as well as in elderly with T2DM compared to healthy elderly. Arterial pressure increases with aging due to several factors, including an increase in the sympathetic tone [49–53]. The sympathetic outflow further increases in the presence of T2DM, which may explain the greater MAP in individuals with T2DM compared to healthy age-matched individuals [60–62]. Tissue BF is precisely determined by the interaction between tissue VC and MAP [113]. Thus, similar baseline values of BF in old versus young individuals could be explained by higher values of MAP along with lower values of VC. The increase in BF after circulatory reperfusion was greater in healthy old individuals than in healthy young individuals, probably due to the greater MAP. However, the presence of T2DM abolishes such a difference, despite this condition further augments MAP. Overall, these findings show that differences or similarities in VC and BF among groups identified at baseline can change after cuff release.

Circadian variations

The mean values of BF at baseline and after circulatory release were similar in the morning compared to the evening in young healthy individuals. This finding is consistent with similar values of VC and MAP between morning and evening in young subjects. Interestingly, lower values of BF at baseline and after circulatory release in the morning compared to the evening were noticed in H50-80, which could only be explained by the lower increase in VC after cuff release since MAP values were similar. While the presence of lower forearm VC at baseline in healthy elderly has already been reported [44], our findings add to the literature that also the subsequent forearm vasodilation is attenuated in the morning in healthy elderly and that such an attenuated vasodilation capacity in the morning impairs BF towards an ischemic area. Since forearm rapid vasodilation is largely endothelium-mediated [121], our finding of blunted VC increment following circulatory reperfusion agrees with previous studies reporting attenuated endothelial function at 6am compared to 9pm assessed through brachial artery FMD [45]. Similar responses were found in older individuals with T2DM, suggesting that T2DM does

not change the normal circadian variation of VC and BF noticed in healthy older individuals. This is also confirmed by our ANOVA test results that reveal no effect of T2DM on VC and BF between H50-80 and T2DM50-80. However, our data reveal a circadian variation of MAP in individuals with T2DM, but not in healthy age-matched individuals. Increases in sympathetic outflow as a consequence of T2DM have been suggested [60–62]. Such an increase in the sympathetic outflow caused by T2DM might be particularly evident in the morning, explaining the higher values of MAP in the morning in individuals with T2DM.

Sex differences

The mean values of BF at baseline and after circulatory release were augmented in men compared to women in young healthy individuals. This finding is consistent with higher values of VC and MAP in men compared to women in young subjects [86]. However, the presence of different baseline values between sexes needs to be taken into account. We have previously shown that these increases in VC and BF after cuff release are similar between men and women when considering the percent increase from baseline values [86], which differed at a young age but not in adulthood. Consistent with this notion, such sex differences seem to disappear with aging, regardless of the presence of T2DM. Indeed, our ANOVA results revealed no effect of sex on BF, VC and MAP between H50-80 and T2DM50-80. Values of BF, VC, and MAP were indeed similar between men and women in the older groups both at baseline and after circulatory reperfusion. Neurovascular regulation differs between sexes and over aging due to several reasons. Resting sympathetic nerve activity is lower in young women compared to young men [86, 122]. There is no correlation between muscle sympathetic nerve activity and vascular resistance in young women, whereas it is present in young men, suggesting a blunted sympathetic vascular transduction in women [89, 122–124]. Indeed, young women show a blunted vasoconstriction in response to sympathetic stimulants [86, 122]. A relaxing effect of oestrogens on vascular smooth muscle has also been suggested in young women [14]. Overall, such factors account for the lower values of VC and arterial pressure in young women compared

to men. However, muscle sympathetic nerve activity increases with aging [49–52]. Following menopause, muscle sympathetic nerve activity in women can exceed that of men [52, 89]. Moreover, there is a decline in oestrogens production in women after menopause [15]. Consequently, women show a steeper increase in arterial pressure with aging, especially after menopause, which may abolish sex differences in adulthood that are otherwise present at a young age [105].

CONCLUSION

Forearm vasodilation following circulatory reperfusion is attenuated in the morning compared to the evening in healthy elderly and such an attenuated vasodilation capacity impairs BF towards an ischemic area. The presence of T2DM does not affect circadian responses in VC and BF compared to those observed in healthy individuals of similar age, but induces circadian variations of MAP, being greater in the morning. There are overt sex differences in VC, BF, and MAP at baseline and after circulatory reperfusion at a young age, being greater in men, which disappear with aging without being affected by T2DM.

CHAPTER 5: SUMMARY AND FINAL CONCLUSION

Highlights:

- the baseline constriction of peripheral vascular tissue is greater in the morning than in the evening, but this is not due to greater sympathetic vasoconstriction responsiveness in the morning.
- this higher morning vasoconstriction at baseline can affect the tissue's ability to supply blood to an ischemic tissue.
- similar sympathetic vasoconstriction responsiveness between morning and evening may explain similar or lower values of central artery stiffness in the morning.
- adults with T2DM report an increase in sympathetic-mediated dilatation capacity on the vascular tissue, which may be a defense mechanism to reduce the central pressor response during sympathetic excitation.

The current literature reveals a lack of information on the circadian variations of some important cardiovascular risk factors. These factors include aortic stiffness, peripheral vasoconstrictor responsiveness, and vasodilation capacity. Furthermore, it is not clear whether the impact of an external stressor capable of activating the sympathetic nervous system could have greater repercussions on the cardiovascular system in the morning than in the evening. Aortic stiffness affects the afterload, work, and oxygen demand of the heart and is a strong predictor of cardiovascular risk. Peripheral vasoconstriction affects pulse pressure and arterial stiffness, and thereby the risk for cardiovascular events by augmenting the cardiac afterload and the formation of acute and chronic vascular lesions. The vasodilation capacity following tissue ischemia is a vital function as it allows to supply blood and oxygen towards an area in need of oxygen. The studies undertaken in this thesis aim to investigate the circadian variations of these risk factors, both at rest and during acute activation of the sympathetic nervous system. Type 2 diabetes is a condition that induces deleterious changes in cardiovascular function, augmenting cardiovascular mortality and morbidity. Therefore, the effect

of diabetes was investigated by comparing the data collected in healthy elderly versus those with diabetes. As a secondary purpose, considering the sex difference in the incidence and prognosis of cardiovascular disease, which worsens to a greater extent in women with diabetes, sex differences were evaluated.

Aortic stiffness assessed via cf-PWV proved not to be increased in the morning compared to the evening at specific times when the cardiovascular risk has been suggested to be significantly different, both at rest and during sympathetic activation. There was no effect of T2DM on circadian variations of cf-PWV in both rest and sympathetic activation conditions. A significant interaction between sex and T2DM was however found during sympathetic activation, but not at rest. While healthy older women show similar aortic stiffness values compared to their male counterparts during acute stress, older women with T2DM reported greater aortic stiffness compared to men with T2DM. This finding agrees with previous findings suggesting that T2DM induces more deleterious effects on cardiovascular health in women than in men, although effects on aortic stiffness are noticeable only during a stressful condition.

Forearm vasodilation following circulatory reperfusion is attenuated in the morning compared to the evening in adults without and with T2DM, and such an attenuated vasodilation capacity impairs blood flow towards an ischemic area. The presence of T2DM does not affect circadian responses in vascular conductance and blood flow compared to those observed in healthy individuals of similar age, but induces circadian variations of arterial pressure, being greater in the morning. There are overt sex differences in vascular conductance, blood flow, and arterial pressure at baseline and after circulatory reperfusion at a young age, being greater in men, which disappear with aging without being affected by T2DM.

The peripheral vasoconstriction triggered by a standardized sympathetic stressor is similar between morning and evening, regardless of the presence of T2DM and reduced baseline VC values in the morning. Moreover, the peripheral vasoconstriction responsiveness is paradoxically blunted in individuals with T2DM than in healthy ones as handgrip-mediated sympathetic activation induced vasodilation on the contralateral forearm in individuals with T2DM and

vasoconstriction in healthy age-matched subjects. This finding highlights a neurovascular response to an external stressor altered by T2DM.

Taken together, our findings seem to suggest that the baseline state of constriction of the peripheral vascular tissue is greater in the morning than in the evening. This affects the capacity of a vascular tissue to dilate and, in turn, the ability to supply blood to an ischemic tissue. The same external stressor capable of activating the sympathetic nervous system induces similar changes in the peripheral vasoconstriction between morning and evening. Thus, the increased state of vascular constriction at baseline in the morning may be consistent with reduced endothelial function and increased sympathetic tone at baseline, but not with greater peripheral constricting responsiveness. Similar sympathetic vasoconstriction responsiveness between morning and evening is a likely factor explaining similar or lower values of central artery stiffness in the morning than in the evening, not only at rest but also during sympathetic excitation. Interestingly, our data reveal a neural response altered by the presence of T2DM. However, paradoxically, the altered response consists of an increase in sympathetic-mediated dilatation capacity on the vascular tissue, and not in an augmented sympathetic-mediated vasoconstriction. This alteration could be a defense mechanism that allows to reduce the central pressor response during sympathetic excitation by dilating peripheral tissues. This mechanism could be a further reason explaining the lack of effects of T2DM on aortic stiffness and vasodilation capacity in individuals with T2DM compared to their healthy counterparts. In contrast, the vasodilation capacity seems to be unaffected by T2DM. Our data reveal and confirm that there are overt sex differences in neurovascular regulation and sympathetic neurovascular transduction, which may change in the presence of T2DM. The presence of T2DM shows sex differences in aortic stiffness that may only be visible during acute stress but not at rest. There are sex overt differences in vasodilation capacity, which are however accounted for differences in body volume. In contrast, the peripheral vasoconstrictor response triggered by an external sympathetic stimulus is similar between sexes regardless of the presence of T2DM.

APPENDIX 1: ESTIMATION OF CAROTID-FEMORAL PULSE WAVE VELOCITY FROM FINGER PHOTOPLETHYSMOGRAPHY SIGNAL.

Pubblished in Physiological Measurement. doi: 10.1088/1361-6579/ac7a8e.

Rationale and clinical significance of the study.

The recording of carotid-femoral pulse wave velocity (cf-PWV) performed during the study described in chapter 2 had several limitations, including the long training time and the operator's skill dependency, the relatively long time needed to perform the measurement, the need to undress the patient to expose the groin, a great variability between operators in the measure of the transit distance, and the inability to obtain continuous beat-to-beat measurements over time. Thus, by taking advantage of the data collected during the study, I used timing features from a finger digital photoplethysmography signal combined with subject height (and other clinical data) to estimate cf-PWV after training regression models using different techniques. The paper shows the potential of exploiting data embedded in pulse waveforms, measurable with simple technologies and artificial intelligence or artificial intelligence-like approaches. The paper also shows the potential of trained models to use data embedded within pulse wave signals for the estimation of physiological cardiovascular parameters. I developed user-friendly software able to estimate the cf-PWV, which addresses the aforementioned limitations of the gold-standard technique. The measure was easier and faster, as data collection and analysis take less than a couple of minutes. It is not necessary to uncover the groin as required for the gold-standard cf-PWV measure since data are taken from the subjects' fingers. The method does not require measuring the pulse transit distance. Full training for novice operators can be provided quickly within approximately one hour. This method has the potential to estimate cf-PWV beat-to-beat and under dynamic conditions, such as during exercise. Estimated cf-PWV were rated as excellent compared to those obtained from the gold-standard technique.

Keywords: Aortic stiffness; Finapres; MATLAB; PPG

ABSTRACT

This project compared a new method to estimate the carotid-femoral pulse wave velocity (cf-PWV) to the gold-standard cf-PWV technique. The cf-PWV was estimated from the pulse transit time (FPS-PTT) calculated by processing the finger photoplethysmographic signal of Finapres (FPS) and subject's height only (brief mode) as well as along with other variables (age, heart rate, arterial pressure, weight; complete mode). Doppler ultrasound cf-PWVs and FPS-PTTs were measured in 90 participants equally divided into 3 groups (18-30; 31-59; 60-79 years). Predictions were performed using multiple linear regressions (MLR) and with the best regression model identified by using MATLAB Regression Learner App. A validation set approach (60 training datasets, 30 testing datasets; VSA) and leave-one-out cross-validation (LOOCV) were used. With MLR, the discrepancies were: -0.01±1.21 m/s (VSA) and 0.001±1.11 m/s (LOOCV) in brief mode; -0.02±0.83 m/s (VSA) and 0.001±0.84 m/s (LOOCV) in complete mode. Using a linear support vector machine model (SVM) in brief mode, the discrepancies were: 0.01±1.19 m/s (VSA) and -0.01±1.06 m/s (LOOCV). Using an Exponential Gaussian process regression model (GPR) in complete mode, the discrepancies were: -0.03±0.79 m/s (VSA) and 0.01±0.75 m/s (LOOCV). The cf-PWV can be estimated by processing the FPS-PTT and subjects' height only, but the inclusion of other variables improves the prediction performance. Predictions through MLR qualify as acceptable in both brief and complete modes. Predictions via linear SVM in brief mode improve but still qualify as acceptable. Interestingly, predictions through Exponential GPR in complete mode improve and qualify as excellent.

INTRODUCTION

The aortic stiffness is an independent predictor of cardiovascular mortality [125]. The non-invasive gold-standard measure to assess the aortic stiffness is the carotid-femoral pulse wave velocity (cf-PWV) measurement [125]. This technique determines the velocity of the blood volume wave propagating over the arterial tree by dividing the pulse transit distance for the pulse transit time between the common carotid artery and the common femoral artery. The cf-PWV assessment has become a common procedure in clinical practice since it can be performed quickly and non-invasively through various techniques and devices [119, 126]. Several cut-off values have been proposed to score the cardiovascular risk according to the subjects' characteristics [127, 128]. However, this technique has several limitations. These include the long training time and the operator's skill dependency, the relatively long time needed to perform the measurement, the need to undress the patient to expose the groin, a great variability between operators in the measure of the transit distance, and the inability to obtain continuous beat-to-beat measurements over time [129].

Previous studies have used the finger photoplethysmographic signal (FPS) to estimate central arterial stiffness. Particularly, a recent study proposed a novel approach to estimate the aortic pulse wave velocity (aPWV), a surrogate index of aortic stiffness related to cf-PWV [130]. This method applies the oscillometric working principle of the Arteriograph device (TensioMed Kft, Budapest, Hungary) to the FPS of the Finapres device (Finapres Medical System BV, The Netherlands) and determines the aortic pulse transit time by detecting specific features on the first- and second-order derivatives of the FPS [130]. Another investigation showed that the PPGAI index, which is also determined by processing the FPS, is strongly correlated to the aortic augmentation index and able to discriminate individuals with augmented arterial stiffness compared to healthy individuals [131]. For the assessment of peripheral arterial stiffness, the transient time from the R wave of ECG signal to the foot of the pressure wave recorded through finger photoplethysmography has been widely used in research as an index of upper limb arterial stiffness [132–134]. Pulse wave velocity measurements by photoplethysmography have also been performed between other points, such as

from ear to finger, ear to toe, and finger to toe [132–135]. Interestingly, the subject's height is proportional to the carotid-femoral length and has been used to estimate the pulse travel distance via mathematical equations [95]. Previous studies have also shown a relationship between the cf-PWV and age [136, 137], heart rate [138], arterial pressure [102, 136], and body weight [139, 140], suggesting that these variables may be co-variants of the cf-PWV. Indeed, these variables have been integrated into mathematical equations to improve the accuracy of the cf-PWV estimation and used to estimate the cf-PWV or its surrogates [95, 137, 141–143].

This project aims to evaluate a new method to estimate the cf-PWV from multiple input variables. It is tested whether the cf-PWV can be estimated from the pulse transit time calculated by processing the FPS signal of Finapres (FPS-PTT) and subjects' height only, the two main variables needed to determine the PWV (time interval and distance, respectively). It is also tested whether the inclusion of other input variables (age, heart rate, arterial pressure, weight) improves the accuracy of the cf-PWV prediction. Predictions are obtained through multiple linear regressions and also by using the best regression model identified with the Regression Learner App of MATLAB (MATLAB, MathWorks, US). Estimated measures will be compared to the gold-standard ones.

MATERIAL AND METHODS

Groups	*18-29 Y.O.*	*30-59 Y.O.*	*60-79 Y.O.*
Age (years)	24.0±2.4	44.8±10.4	68.1±4.9
Weight (Kg)	67.9±11.3	71.7±9.8	77.1±8.5
Height (cm)	1.74±0.10	1.71±0.06	1.70±0.07
Systolic BP (mmHg)	114.7±9.2	129.1±10.9	137.9±10.4
Diastolic BP (mmHg)	67.4±6.2	74.4±8.3	81.2±6.9
Resting heart rate (HR)	60.8±9.5	64.5±8.9	67.2±6.8
FPS-PTT (s)	0.17±0.01	0.15±0.02	0.12±0.02
Doppler cf-PWV (m/s)	5.4±0.6	6.9±1.1	8.8±1.4

Table 1. Characteristics of groups (mean ± standard deviation; 15 men and 15 women within each group).

Measures were performed in 90 participants meeting inclusion (>18 years old) and exclusion criteria (atrial fibrillation and cardiac valve disease, not in sinus rhythm, pacemaker-dependent, pregnancy, BMI>30 kg/m2, known significant carotid or femoral artery stenosis, impalpable arterial pulse) [119]. Subjects were divided into 3 groups by age as shown in Table 1 (18-30 y.o.; 31-59 y.o.; 60-79 y.o.; 15 men and 15 women within each group). Personal data (age, weight, height) were recorded before starting the test. Subjects were connected to the input channel of the 3-lead electrocardiograph integrated into the ultrasound scanner (LOGIQ S7 pro, GE, Milwaukee, USA) through the use of skin electrodes. Moreover, subjects were instrumented with the beat-by-beat finger blood pressure monitoring system Finapres on the third medial phalanx of the right hand. The Finapres analog output was connected to an analog-to-digital converter (ESP32, AZDelivery, Germany) sampling at 1 kHz. Finapres data were saved into .txt files. The cf-PWV assessment was performed complying strictly with the recommendations on user procedures previously indicated [95]. After 10 minutes of supine and quiet rest, 3 arterial pressure measurements were taken using the Riva-Rocci method on the left arm and averaged to obtain systolic (SAP) and diastolic (DAP) arterial pressure values, whereas the resting heart rate (HR) was read from the Finapres serial monitor. Then, the following measures were performed.

FPS-derived pulse transit time

The FPS-PTT was calculated complying strictly with the procedure previously indicated by Pilt et al. [130] with a slight modification (details below). The algorithm proposed by Pilt et al. has been explained in detail in their article [130] and has been integrated into a MATLAB sketch by ourselves for being used in our project. The software has been implemented by ourselves with a user-friendly graphical interface to graphically detect the FPS-PPT (Figure 1) to further simplify the signal analysis. The procedure for calculating the FPS-PTT is as follows. After running the MATLAB sketch, a pop-up window allows to upload the .txt file containing the numerical data of the Finapres signal to be analyzed. The software automatically filters the data through high- and low-pass filters. Cut-off frequencies

are 0.1Hz and 30Hz, respectively. Then, a graphical interface showing the first- and second-order derivatives of FPS appears on the screen. The user needs to click the pointer on the first zero-crossing point of the first-order derivative and on the second valley of the second-order derivative to determine the FPS-PTT, as shown in Figure 1. The resolution to measure the time delay between the two points is 1 ms. The graphical interface shows the entire signal divided into subsequent 3-second windows to allow 15 consecutive measurements. When the 15 measures are completed, the software returns the average value of FPS-PTT on the screen. The original algorithm proposed by Pilt et. al [130] requires selecting the first zero-crossing point of the first-order derivative and, if visible, the third zero-crossing point of the first-order derivative. If the latter is not visible, the second valley of the second-order derivative needs to be selected. Indeed, the FPS-PTT may slightly change with aging and the third zero-crossing point of the first-order derivative may not be visible [130] (Figure 2). Our modification consists of the standardization of the selection of the second valley of the second-order derivative across all subjects, even if the third zero-crossing point of the first-order derivative is visible. Our modification consists of the standardization of the selection of the second valley of the second-order derivative across all subjects, even if the third zero-crossing point of the first-order derivative is visible, since these two points are almost coincident (Figure 2, left panel). This change also simplifies signal analysis and technique teaching.

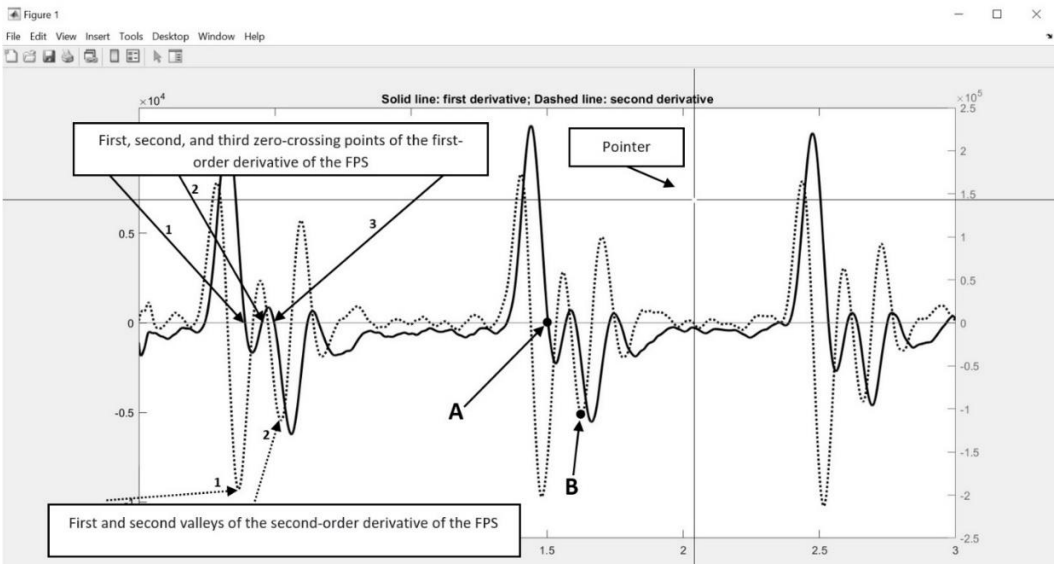

Figure 1. The figure shows the graphical interface with the first- (solid line) and second- (dashed line) order derivatives of the finger photoplethysmographic signal (FPS) with their zero-crossing points and valleys. To determine the FPS-derived pulse transit time, the software requires to click the pointer on the first zero-crossing point of the first-order derivative (A) and on the second valley of the second-order derivative (B).

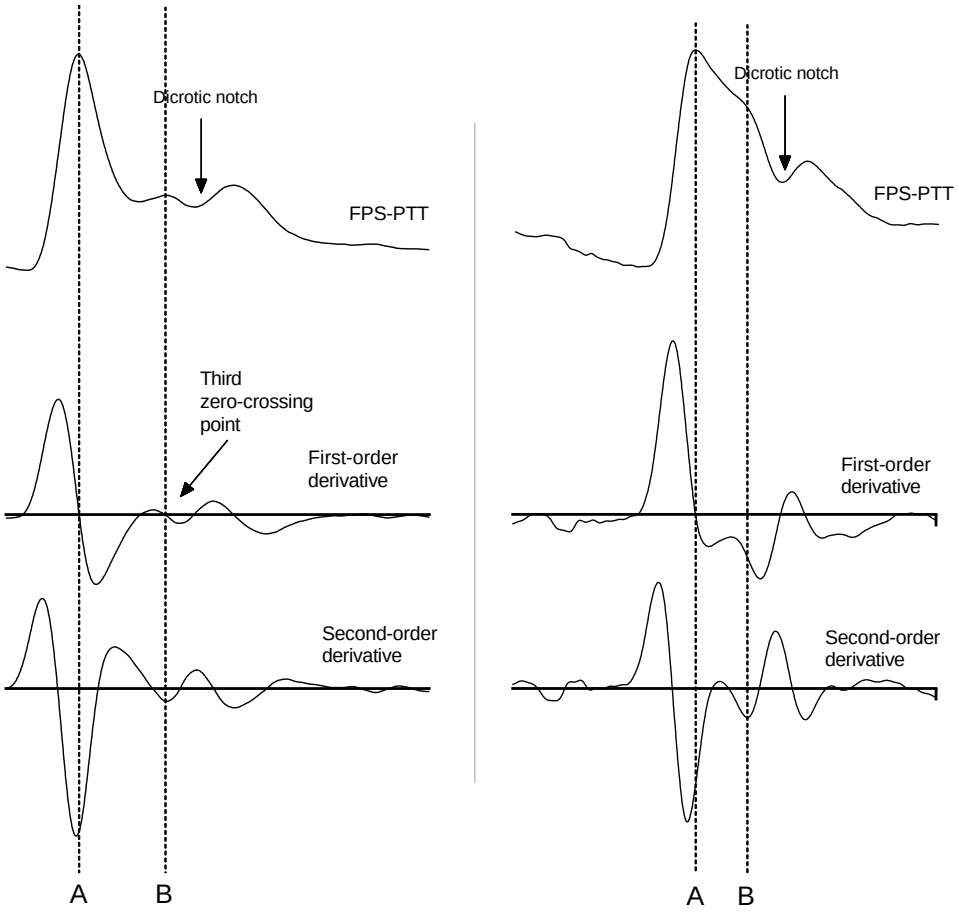

Figure 2. FPS-PTT, first-order derivative of FPS-PTT, and second-order derivative of FPS-PTT on a 20 years old men (left panel) and a 65 years old men (right panel). 'A' represents the first zero-crossing point of the first-order derivative, while 'B' represents the second valley of the second-order derivative. The third zero-crossing point of the first-order derivative is visible on the young men, but not on the older one.

Doppler Ultrasound cf-PWV measure

Details and graphical description about this procedure have been previously described [95, 97]. Briefly, scanning of the carotid artery at the supraclavicular level followed by another scanning of the common femoral artery in the groin were performed in B-mode using the pulsed Doppler function of our ultrasound scanner with a Linear Array (6.6 MHZ) probe synchronized with ECG. The pulse transit

times at the carotid and femoral arteries were identified by measuring the time elapsed from the R peak of the ECG signal to the foot of the Doppler flow waves at the carotid and femoral recordings, respectively, as graphically shown in the paper by Calabria et al. [97]. The foot of the Doppler flow wave defines the point where the steep rise of the waveform begins. Pulse transit times were measured offline using the proprietary software integrated into our ultrasound scanner. The software returns the time delay between two points of interest after positioning two movable cursors in correspondence of such points. The resolution to measure the time delay between the two points is 1 ms. The average values of the pulse transit times at the carotid and femoral arteries over 15 subsequent cycles were calculated. The pulse transit time was calculated by subtracting the average pulse transit time at the carotid artery from the average pulse transit time at the femoral artery [95]. The cf-PWV was calculated as 0.8 times the direct body surface distance from the common carotid artery to the common femoral artery at the groin divided by the pulse transit time [95]. Ultrasound measures were performed by an expert sonographer with >500 hours of experience.

Validation methods

Two validation methods were used. First of all, we proceeded with a validation set approach (VSA). Data from 60 random participants (20 per group) were used for training, whereas the data of the other 30 participants were used for testing. Importantly, the training and testing datasets were determined once and then used to train and test all regression models. Secondly, we proceeded with a leave-one-out cross-validation (LOOCV). Repeatedly, each subject was excluded from the complete dataset, model training was performed with the data of the other 89 subjects and used to predict the cf-PWV of the excluded subject.

Multiple linear regression analysis

The relationship between independent variables (FPS-PTT, height, age, heart rate, weight, and systolic and diastolic arterial pressure) and the dependent

variable (Doppler cf-PWV) was assessed by multiple linear regressions. It was obtained a mathematical equation to predict the cf-PWV from FPS-PTT and subjects' height only (brief mode), as well as another equation by also including age, heart rate, arterial pressure, weight as input variables (complete mode).

Analysis via MATLAB Regression Learner App

The Regression Learner App of MATLAB was used to assess and choose the multiple regression model with the best performance in predicting the cf-PWV. After entering input and target data, this App trains a wide range of regression models and compares their validation errors side-by-side. Thus, the regression model with the best performance can be chosen, exported, and used to make predictions by entering new input data via MATLAB code. It was chosen the regression model with the best performance using FPS-PTT and subjects' height only as inputs, as well as the best one using all input variables.

Statistics

The relationship between each independent variable and the Doppler cf-PWVs was assessed via linear regression. Predicted cf-PWVs were compared with the Doppler cf-PWVs through Bland-Altman plots and linear regression. Data analysis was performed by using MATLAB. GraphPad Prism 8 (GraphPad Software, San Diego, United States) was used for statistical analysis and graphs. To improve the fairness of the comparison between previous results and our own, we repeated the comparison between estimated cf-PWV and Doppler cf-PWV on a subset of subjects reporting similar features to those recruited in similar previous studies. Once the target features (number of subjects, sex distribution, age range, mean age) to be obtained in the new group were set, the subjects to be included were randomly chosen from the full dataset through MATLAB.

RESULTS

The relationship between each independent variable and the Doppler cf-PWVs along with their coefficient of determination is shown in Figure 3.

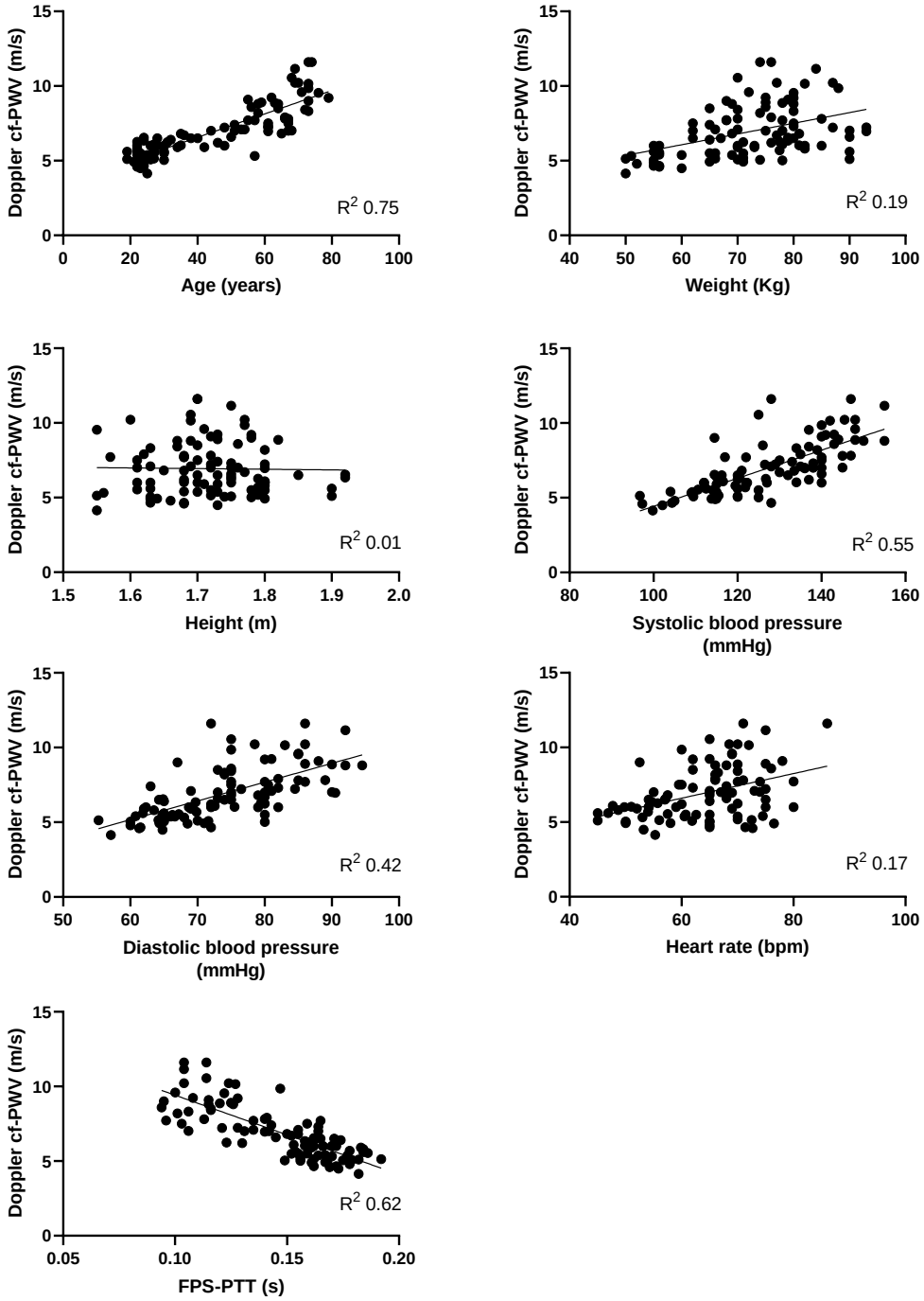

Figure 3. Relationship between input variables and cf-PWV.

Multiple regression analysis

By using VSA, the bias of the technique was 0.01 m/s and the SD of bias was 1.21 m/s in the brief mode, whereas the bias of the technique was -0.02 m/s and the SD of bias was 0.83 m/s in the complete mode. The regression equations obtained were:

- $cfPWV = +12.097 + height * 1.748 - FFSPTT * 55.624$
- $cfPWV = -2.572 + age * 0.056 - weight * 0.012 + SIS * 0.028 - DIA * 0.015 + HR * 0.013 + height * 4.02 - FFSPTT * 15.45$

By using LOOCV, the bias of the technique was 0.001 m/s and the SD of bias was 1.11 m/s in the brief mode, whereas the bias of the technique was 0.001 m/s and the SD of bias was 0.84 m/s in the complete mode.

Analysis via MATLAB Regression Learner App

With VSA, the best regression model for the brief mode was a linear support vector machine, which led to a bias between the techniques of 0.01 m/s and a SD of bias of 1.19 m/s. The best regression model for the complete mode was an Exponential Gaussian process regression, which led to a bias between the techniques of -0.03 m/s and a SD of bias of 0.79 m/s.

With LOOCV, for each subject, the best regression models were Linear support vector machine and Exponential Gaussian process regression for the brief and complete modes, respectively. By using LOOCV, the bias of the technique was -0.01 m/s and the SD of bias was 1.06 m/s in the brief mode, whereas the bias of the technique was 0.01 m/s and the SD of bias was 0.75 m/s in the complete mode.

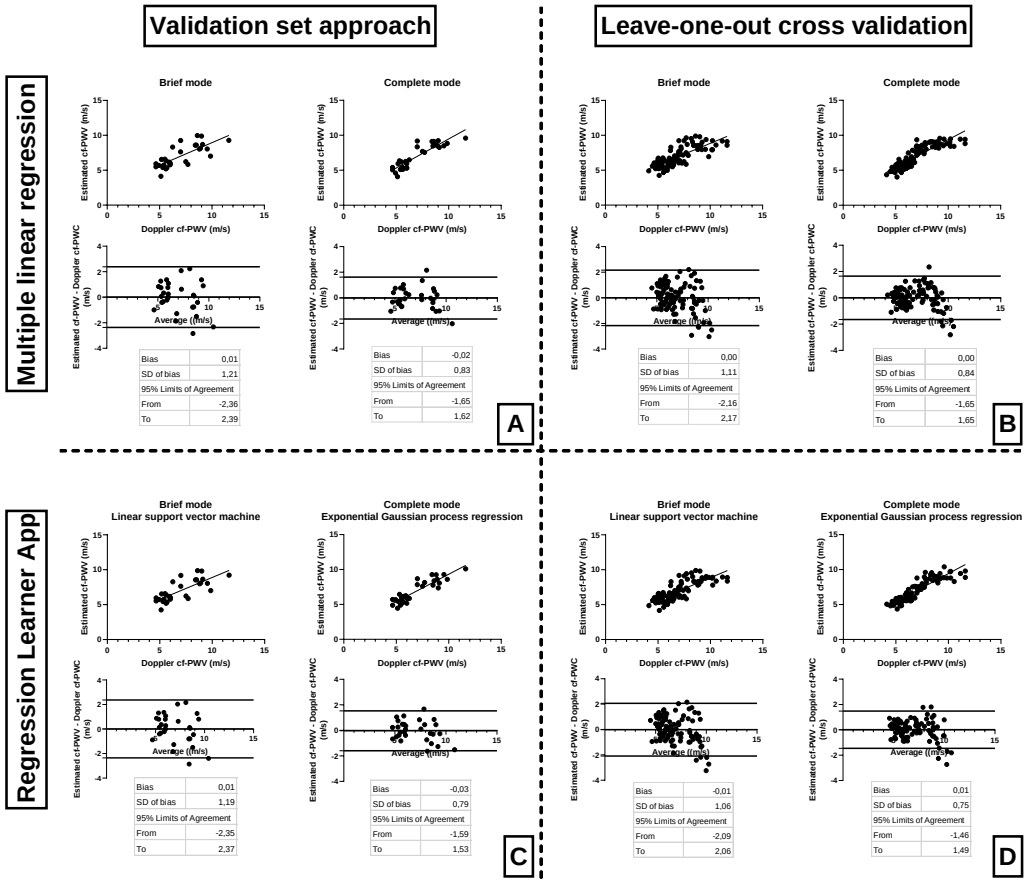

Figure 4. Comparison of the estimated cf-PWVs with the gold-standard measures. The cf-PWVs were estimated with multiple linear regression (A, B) and with the best regression model identified with MATLAB regression learner App (C, D) with a validation set approach (A, C; n=60 training dataset and n=30 testing dataset) and leave-one-out cross-validation (B, D).

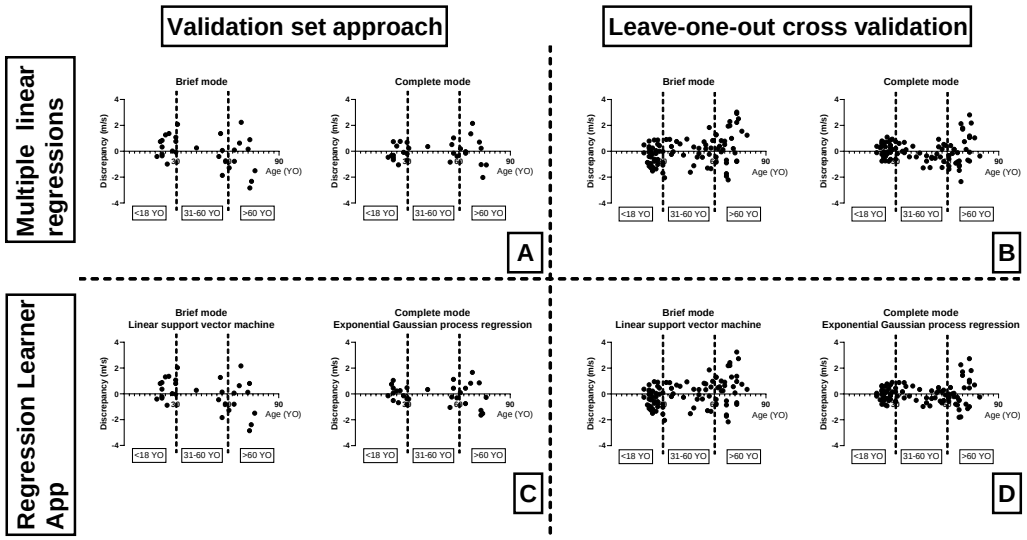

Figure 5. Discrepancy between the precicted cf-PWV and the gold-standard cf-PWV measures over age.

Subgroup results

To improve the comparison between the results by Pilt et al [130] and our own, we created a random subset of 23 healthy subjects (18 men and 5 women; age range: 20-64 y.o .; mean age: 34.3±12.5 y.o.) from the full dataset. In this subgroup, the discrepancies between estimated cf-PWVs and Doppler cf-PWVs are as follows (bias±SD; MLR and VSA: brief mode 0.23±0.97 m/s, complete mode -0.04±0.62; MLR and LOOCV: brief mode 0.11±0.86, complete mode 0.07±0.61; Linear support vector machine and VSA: brief mode 0.22±0.95; Exponential Gaussian process regression and VSA: complete mode 0.05±0.61; Linear support vector machine and LOOCV: brief mode 0.05±0.85; Exponential Gaussian process regression and LOOCV: complete mode -0.01±0.54).

DISCUSSION

We sought to evaluate a new method to estimate the cf-PWV from multiple variables. We tested whether the cf-PWV can be estimated from the pulse transit time (FPS-PTT) calculated by processing the finger photoplethysmographic signal

of Finapres and the subjects' height only (brief mode). We also tested whether the inclusion of other input variables (age, heart rate, arterial pressure, weight; complete mode) improves the accuracy in the cf-PWV prediction. Predictions were made using multiple linear regressions, as well as with the best regression model identified with the MATLAB Regression Learner App. We used a VSA (60 subjects for training; 30 subjects for testing), as well as LOOCV (89 subjects for training; 1 subject for testing) as validation methods. According to the guidelines for validation of non-invasive arterial pulse wave velocity [119], the accuracy of the test device is scored as 'excellent' when the bias from the gold standard measure is <0.5 m/s and the SD is <0.8 m/s, and 'acceptable' when the bias from the gold standard measure is <1.0 m/s and the SD is <1.5 m/s.

Multiple linear regression is a simple, widely-used function to predict a target variable from independent variables through a mathematical equation. This function is integrated into user-friendly calculation systems such as Microsoft Excel (Microsoft Corporation, US). The Regression Learner App of MATLAB is a powerful tool that compares multiple regression models and allows to choose the one with the best performance. The best model is exported as a MATLAB file and used along with new input data to make predictions in new subjects via MATLAB code. Although the Regression Learner App could find regression models with greater performance than multiple linear regressions, it does not provide mathematical equations and requires substantial MATLAB coding skills to use the exported models and make predictions with new input data. Therefore, the use of multiple linear regressions would allow the use of the technique to a wider audience. The VSA provides a unique model or equation based on a portion of available data. The use of LOOCV allows the use of a larger training dataset compared to VSA since it repeatedly fits a model to a dataset that contains a number of observations equal to the total sample size minus 1. Furthermore, the use of LOOCV allows a final comparison of the techniques on a greater number of data points compared to VSA.

We used a new approach to calculate a variable related to the carotid-femoral pulse transit time, the FPS-PTT. It was calculated by applying the oscillometric working principle of Arteriograph to the FPS of Finapres as recently

proposed by Pilt et al [130]. The oscillometric algorithm of Arteriograph assumes to determine the aortic pulse transit time by detecting the time elapsed between the first wave ejected from the left ventricle to the aortic root and its reflection from the aortic bifurcation as the second systolic wave [144, 145]. Subsequent research has questioned the existence of a discrete arterial reflection site and supported the notion of the presence of an effective reflection site that conceptually includes the integration of all scattered reflections that take place over the arterial tree, without connecting it to a precise anatomical location [145, 146]. Such an effective reflection site is linked to the path traveled by the diffuse waves across the various segments of the arterial tree, whose length shows a certain degree of proportionality with the body height [95, 145, 147]. Arteriograph underwent both noninvasive [126, 144, 148–151] and invasive comparisons [152] against gold-standard cf-PWV methods, although there has been some debate regarding whether it measures the aortic stiffness directly or indirectly by measuring the axillo-brachial stiffness [153]. Interestingly, as shown in Figure 3, the FPS-PTT shows a relationship with the cf-PWV and tends to decrease with aging. This would be consistent with a faster pulse wave velocity in the elderly compared to young individuals [136, 137]. The algorithm proposed by Pilt et al.[130] has been integrated and implemented into MATLAB software by ourselves. The software provides a graphical interface to quickly determine the FPS-PTT by clicking on specific features of the first- and second-derivative of the FPS with the mouse pointer as shown in Figure 1. Specifically, the first zero-crossing point of the first-order derivative and the second valley of the second-order derivative need to be selected as these points are visible across all subjects regardless of the age (Figure 2). The graphical detection of the FPS-PTT allows fast training to inexperienced users with little operators' skill dependency.

Linear multiple regression analysis

As shown in Figure 4, the cf-PWV predictions from FPS-PTT and subjects' height only via VSA qualify as acceptable. The bias between the gold-standard values of cf-PWV and those predicted in 30 new subjects is close to 0 m/s. This

result is relevant as it arises from the interaction between a time interval and a length only, in a group of 30 new test subjects not used to develop the regression model. The carotid-femoral length has been shown to be proportional to subjects' height [95]. This result implies that the FPS-PTT also be proportional to the carotid-femoral pulse transit time. The inclusion of other input variables (age, heart rate, arterial pressure, weight) improves the accuracy in the cf-PWV prediction via VSA compared to the brief mode. Indeed, the SD of bias decreases from 1.21 to 0.83 m/s. The complete mode still qualifies the prediction as acceptable, however, such results are close to the threshold to qualify the prediction as excellent (SD=0.80 m/s). The cf-PWV prediction in the brief mode slightly improves by using LOOCV compared to the VSA, although it still qualifies as acceptable. In the complete mode, the cf-PWV predictions with LOOCV compared to the VSA remain similar, suggesting that a multiple regression model fitted on more than 60 subjects does not necessarily improve accuracy in the cf-PWV prediction.

Analysis via MATLAB Regression Learner App

The regression models identified by MATLAB's Regression Learner App have improved cf-PWV prediction performance compared to multiple linear regressions, but only to a minimal extent. The cf-PWV prediction in brief mode with a Linear support vector machine model still qualifies as acceptable both with a VSA and LOOCV. In the cf-PWV prediction in complete mode with an Exponential Gaussian process regression model and VSA, although the bias does not change markedly, the SD of bias diminishes from 0.83 to 0.79 m/s. With the use of LOOCV, the SD of bias diminishes to 0.75 m/s. Under such circumstances, the cf-PWV predictions would qualify as excellent. With the threshold for the 'excellent' set at an SD of bias of 0.80, however, it might be more prudent to qualify the predictions between excellent and acceptable in practice. Therefore, the use of the MATLAB Regression Learner App has identified regression models with better performance than multiple linear regressions in predicting the cf-PWV. Despite the difference in prediction being pretty small, such a difference could improve the

qualification of the prediction performance from acceptable to excellent in some circumstances.

Comparison with previous device validation results

In the study by Pilt et al. [130], the aPWV calculated by processing the Finapres signal was compared to the aPWV of Arteriograph on 23 subjects (age distribution not indicated), showing a bias between the techniques of 0.07 m/s and a SD bias of 0.51 m/s. Consistent with the previous study, our results from the full dataset show a small bias between the techniques but a slightly higher SD of bias. This discrepancy might be due to the different age distribution of the subjects. As shown in Figure 5, the discrepancy between the techniques is much greater in subjects older than 59 and the inclusion of such subjects in the analysis may therefore increase the SD of bias. Indeed, the SD of bias diminished when we repeated comparisons on a subset of subjects with similar ages to those included in the study by Pilt et al. [130], showing values of SD ranging from 0.54 and 0.97 m/s depending on the condition. Conversely, the bias of the technique slightly increased in this subgroup in a range between -0.01 and 0.23 m/s depending on the condition. A previous study tested the agreement of the cf-PWV values assessed via Doppler Ultrasound against those assessed via the Complior device (Artech Medical, Pantin, France) in 40 subjects [97]. The bias between the devices was 0.13 m/s, the limits of agreement were approximately (graphic data provided only) by 2 m/s (SD approximately 1 m/s), and R=0.91. Regardless of the regression model and validation method chosen, our technique in complete mode provided a greater agreement with the Doppler cf-PWV values. At values higher than 8-10 m/s, however, our predicted cf-PWVs appear to be underestimated and a wider scatter is present. These trends were also found while comparing Arteriograph to Sphygmocor [148] and to Complior [152]. The reasons responsible for such behaviors at higher PWV values have not been elucidated [144, 148], however, it has been speculated that these might derive from the fact that the aorta is to a variable degree increasing in length with aging. The ascending aortic length increases with aging up to double from 20 to 80 years of age, whereas the length of the other aortic segments increases or decreases to a lower extent [154]. The impact of age-related increases of the ascending aorta on cf-PWV is small because this

tract is not considered in the carotid-femoral length [154]. However, it could affect the PWV assessed through the oscillometric algorithm of Arteriograph, because this method considers the pulse transit time from the left ventricle outflow tract to an effective reflection site conceptually located after the heart, over the arterial tree [144]. Any elongations of the aorta would result in a longer pulse transit time and, consequently, in an underestimation of velocity. Between-equipment divergences in the PWV calculation are well known and accepted across devices and have been mainly attributed to differences in calculating the travel distance rather than to differences in calculating the transit time [150]. The need to moderate any results to the device used and to use the same device for repeated measurements has indeed been suggested [150]. A detailed review between the agreements of different commercial devices for measuring cf-PWV is reported in the recent paper by Milan et al. [149].

Strengths and limitations of the technique

The strengths of our method are manifold. It adds important functionality to Finapres, a device commonly found in physiology laboratories. The bias against the gold-standard measure is close to 0 regardless of the mode used. As shown in Table 2, our data reveal no overt sex differences. Data collection and analysis are simple to perform and take less than a couple of minutes. It is not necessary to uncover the groin as required for the gold-standard cf-PWV measure since data are taken from the subjects' fingers. The method does not require measuring the pulse transit distance. Full training to novice operators can be provided quickly within approximately one hour. This method has the potential to estimate cf-PWV beat-to-beat and under dynamic conditions, such as during exercise. As limitations, the software MATLAB and MATLAB coding knowledge are required to determine the FPS-PTT and to make predictions with MATLAB models. Moreover, this study compared the estimated cf-PWV values to the gold-standard ones in healthy subjects only. Further verification using multi-center data and data in other cohorts are required before considering this technique valid for use in research or clinical practice.

			Men	*Women*	*Men vs Women (p-value)*
VSA	Brief mode	MLR	-0.1±1.4	0.1±1.0	*p=0.58*
		SVM	-0.1±1.4	0.2±1.0	*p=0.53*
	Complete mode	MLR	0.1±0.8	-0.1±0.8	*p=0.68*
		GPR	0.0±0.8	0.0±0.8	*p=0.94*
LOOCV	Brief mode	MLR	-0.1±1.2	0.1±1.1	*p=0.66*
		SVM	0.1±1.4	0.2±1.4	*p=0.65*
	Complete mode	MLR	-0.1±0.9	0.1±0.8	*p=0.46*
		GPR	0.0±0.8	0.0±0.8	*p=0.90*

Table 2. Discrepancy (m/s) between the precicted cf-PWV and the gold-standard cf-PWV measures in both sexes (MLR: multiple linear regression; SVM: support vector machine; GPR: gaussian process regression).

Conclusion

Our data suggest that the cf-PWV can be estimated through the FPS-PTT and subjects' height only, showing an acceptable agreement compared to the gold-standard Doppler cf-PWV measure. The inclusion of other variables (age, heart rate, arterial pressure, weight) improves the accuracy in the cf-PWV estimation up to excellent according to the regression model chosen. Predictions through the use of multiple linear regression qualify as acceptable in both brief and complete mode. The use of MATLAB's Regression Learner App has identified regression models with greater performance than multiple linear regressions. The cf-PWV predictions improve using a linear support vector machine model in the brief mode, despite predictions still qualify as acceptable. Interestingly, cf-PWV predictions via the Exponential Gaussian process regression model improve in the complete mode, qualifying as excellent via both VSA and LOOCV.

APPENDIX 2: SYMPATHETIC-MEDIATED BLUNTING OF FOREARM VASODILATION IS SIMILAR BETWEEN YOUNG MEN AND WOMEN.

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Rationale and significance of the study.

The three main studies undertaken during my PhD investigated cardiovascular function at rest and during acute sympathetic stimulation. Several techniques can be used to non-invasively stimulate the sympathetic nervous system, including handgrip-mediated activation of muscle chemoreflex, cold pressor test, lower body suction, and mental arithmetic task. The experiments were initially designed with the cold pressor test stress as a sympathetic stimulant. The test consists of dipping the subjects' feet in a slurry of water and ice for several minutes. However, it was unclear whether this stimulant was free-of-risk for an elderly population with and without diabetes. Therefore, the effects of this stimulant were preliminarily tested in a young population, analyzing the interaction between the capacity of vasodilation and vasoconstriction between sexes. Indeed, although the control of vascular conductance in vivo is based on a continuous balance between sympathetic vasoconstriction and metabolic vasodilation, the preponderance of previous studies has investigated these controls separately and little is known about their interaction. The findings add important pieces to the current knowledge on neurovascular regulation, showing that although women report different vasodilation and vasoconstriction characteristics compared to men when these are studied separately, as previously shown in the scientific literature, the sex differences disappear when these controls interact. These findings, therefore, support the use of an integrative approach in physiology research, as well as the importance of normalizing data to subjects' features. The cold pressor test induced a strong pressor response in young people and was rated as very painful, proving to be worrisome for use in an elderly population. Therefore, the results of this study led to the use of handgrip exercise instead of the cold pressor test in the three main studies undertaken in my PhD.

Keywords: endothelium; rapid vasodilation; cold pressor test.

ABSTRACT

The in-vivo regulation of vascular conductance (VC) is a continuous balance between endothelial vasodilation and sympathetic vasoconstriction. Although women may report blunted sympathetic vasoconstriction along with higher endothelial vasodilation than men, it is currently unknown whether the interaction between vasoconstriction and vasodilation leads to different regulation of VC between sexes. This study assessed sex differences in sympathetic-mediated blunting of endothelial vasodilation after a brief period of ischemia and whether any restriction of vasodilation blunts tissue blood flow (BF) and re-oxygenation. 13 young women and 12 young men underwent two 5-minute forearm circulatory occlusions followed by reperfusion, one in basal conditions and the other during cold pressor test-induced sympathetic activation (SYMP). Brachial artery diameter and BF, mean arterial pressure, total peripheral resistance (TPR), and thenar eminence oxygenation were collected. Percent changes normalized to baseline values of forearm VC, brachial artery BF and flow-mediated dilation (FMD), TPR, and hand oxygenation after circulatory reperfusion were calculated. TPR increased during SYMP in men ($p=0.019$) but not in women ($p=0.967$). Women showed a greater brachial artery FMD than men ($p=0.004$) at rest, but sex differences disappeared after normalization to shear rate and baseline diameter ($p>0.11$). The percent increases from baseline of peak and average forearm VC after circulatory reperfusion did not differ between sexes in basal conditions ($p>0.98$) or during SYMP ($p>0.97$), and were restrained by SYMP similarly in both sexes ($p<0.003$) without impairing the hand re-oxygenation ($p>0.08$) or average hyperemic response ($p>0.09$). Although women may report blunted sympathetic vasoconstriction than men when assessed separately, the similar sympathetic-mediated restriction of vasodilation suggests a similar dynamic regulation of VC between sexes. SYMP-mediated restrictions of the normal forearm vasodilation do not impair the average hyperemic response and hand re-oxygenation in both sexes.

INTRODUCTION

Sex differences in sympathetic neurovascular regulation have been described [89, 122, 155]. In young individuals, muscle sympathetic nerve activity (MSNA) appears to be lower in women compared to men at rest [122]. MSNA seems to be correlated to peripheral vascular resistance in young men, but not in young women [89, 122, 123]. Sympathetic stimulants have been shown to induce less vasoconstriction in women compared to men despite similar MSNA increments [122]. The previous findings support the notion that the transduction of sympathetic activity into vascular resistance is blunted in young women [89, 122, 123]. Sympathetic neurovascular transduction has been proposed to differ between sexes due to multiple factors, including a different sensitivity and distribution of post-junctional α-adrenergic and β-adrenergic receptors [155, 156]. It has been postulated that β-adrenergic vasodilator mechanisms offset α-adrenergic vasoconstriction in women compared to men [157]. When β-adrenergic (vasodilation) activity was blocked, sex differences in resting vasoconstriction responsiveness were abolished [156]. Differences in the type and quantity of neurotransmitters released from the sympathetic nervous system have also been suggested to account for sex differences in neurovascular modulation [15]. Interestingly, as excellently described by Hissen et al [89], the previous findings supporting a blunted vascular transduction of sympathetic activity in women are not universal and strongly depend on the approach used to assess vascular transduction. When sex differences are investigated on a beat-by-beat basis at rest, the preponderance of the previous studies has suggested similar levels of sympathetic vascular transduction between young men and women [89]. Women also seem to have higher endothelial-mediated vasodilation compared to men as suggested by their greater artery flow-mediated vasodilation (FMD) [158]. A key role for oestrogens in relaxing vascular smooth muscle has been suggested in women [14]. The previous research, however, has investigated sex differences in vasodilator and vasoconstrictor responses separately. This approach does not define how these controls interact and how the final neurovascular regulation differs between sexes in controlling vascular conductance (VC) and blood flow (BF) in-vivo. The in-vivo regulation of VC is indeed a dynamic process, involving the

continuous balance between sympathetic vasoconstriction and endothelial-mediated vasodilation [113]. The fine regulation of VC is essential for regulating tissue BF and systemic blood pressure [159].

Sudden vasodilation of a vascular tissue following a period of ischemia provides a fast blood supply to an area in need of oxygen [160]. This takes on particular importance where oxygen-requiring tissues are vital. Considering the remarkable difference between sexes in the prevalence and harshness of cardiovascular disease [14, 15, 155], the identification of sex differences in neurovascular modulation is pertinent as these may provide new insights into cardiovascular medicine. The identification of factors that affect vascular health in one sex can lead to the development of translational studies and therapies to be applied to the other sex, as well as to a differentiated treatment between sexes [161]. This study aims to identify sex differences in the acute sympathetic activation (SYMP)-mediated blunting of forearm vasodilation after a brief period of ischemia to assess the interaction between sympathetic vasoconstriction and endothelial-mediated vasodilation in both sexes. Additionally, this study aims to investigate whether any SYMP-mediated restriction of vasodilation blunts tissue blood flow (BF) and re-oxygenation after circulatory reperfusion in both sexes. According to the current literature, women may have lower sympathetic vasoconstriction as well as higher endothelial-mediated vasodilation compared to men [15]. Therefore, it seems reasonable to hypothesize that any attenuation of vasodilation via SYMP is lower in women compared to men. Moreover, it is hypothesized that SYMP impairs the normal tissue BF and re-oxygenation after circulatory reperfusion in both sexes, but to a greater extent in men compared to women.

MATERIAL AND METHODS

Participants

25 young, healthy, non-smoker, recreationally active individuals were recruited for this study (Table 1). There were 12 men and 13 women. Participants met the inclusion (absence of any muscle-skeletal, metabolic, cardiovascular, and

respiratory disease; between 18 and 25 years of age;) and exclusion (BMI≥28 kg/m^2; diabetes mellitus; hypertensive disorders; use of any drug altering the cardiovascular response to SYMP; family history of premature cardiovascular disease) criteria. Women were not on contraceptives and were tested during the early follicular phase (days 1 to 7) of the menstrual cycle, according to the current recommendations for the assessment of FMD in humans [162, 163]. This phase offers the lowest attainable levels of estrogen and progesterone, in which hormone levels and artery FMD in women are comparable to those of men [16]. All experiments were performed in the morning (at around 10.00 AM) in a quiet and temperature-controlled room (~22°C). Participants were fasting and were asked to abstain from alcohol and caffeine in the 48 hours before the tests. The number of subjects was calculated with an a priori power analysis (GPower 3.1.9.7; Universität Düsseldorf, Germany) for an F test (ANOVA, repeated measures, within-between interaction), partial eta squared of 0.20, statistical power (1−β) of 0.80, level of significance of 0.05. This analysis suggested the need for 7 men and 7 women to assess the sympathetic-mediated blunting of vasodilation. The effect size was calculated according to the different magnitude of sympathetic-mediated restriction of leg VC between sexes in response to the cold pressor test (CPT) [164]. The Ethics Board of the University of Verona approved all procedures involving human subjects (3293CESC). Each participant provided written informed consent before being involved in any test.

	Males (n=12)	Females (n=13)	Males vs Females (p-value)
Age (years old)	25.6±3.7	23.8±2.6	p=0.04
Height (cm)	173.6±5.5	163.8±5.5	p=0.0002
Weight (Kg)	75.92±5.16	56.77±5.05	p<0.0001
Baseline mean arterial pressure (mmHg)	79.9±7.6	75.9±6.3	p=0.17
Baseline brachial artery diameter (cm)	0.39±0.07	0.31±0.04	p=0.002
Baseline brachial blood flow (mL/min)	85.3±32.9	49.9±26.9	p=0.01
Baseline vascular conductance (mL/min/mmHg)	1.04±0.47	0.66±0.35	p=0.03
Baseline heart rate (bpm)	67.5±6.8	66.0±16.1	p=0.85

Table 1. Characteristics of subjects involved in the study.

Subject monitoring

Participants lay supine with their knees bent over the rim of the bed during the experiment. Their right arm was extended for ultrasound measurements. Subjects were fitted with an automatic blood pressure monitor (Tango+, SunTech Medical, Morrisville, NC; USA) at the heart level on their left arm. Subjects were also equipped with a beat-by-beat finger blood pressure monitoring system (Portapres; Finapres Medical System BV, The Netherlands) on their left hand to measure the mean arterial pressure (MAP). The beat-by-beat finger blood pressure system was calibrated with the automatic sphygmomanometer recording the brachial blood pressure. Participants were also instrumented with the 3-lead electrocardiograph (ECG) of the Ultrasound Device (LOGIQ S7 pro, GE, Milwaukee, USA). Near-infrared spectroscopy (NIRS; OxiplexTS, ISS, USA) was used to assess hand oxygenation. NIRS probe was placed on the thenar eminence of the right hand and was completely covered to ensure that environmental light could not reach the probe. A pressure cuff was placed around the right forearm, distal to the imaged artery, 2 cm below the flexion point of the elbow. The cuff was

inflated with a rapid cuff inflator (Hokanson, Bellevue, USA) >50 mmHg above the systolic blood pressure. The cuff could be deflated within approximately 300 ms. While subjects were asked to stay relaxed and breathe regularly, the right brachial artery was scanned via pulsed Doppler ultrasonography to simultaneously detect mean blood velocity and measure the brachial artery diameter. The probe location was marked to evaluate the same artery section at rest and during SYMP. Data were collected using a 4.4 MHz probe with a 60° angle of insonation. The ultrasound gate was adjusted to examine the whole artery width. The sample volume was aligned and regulated according to vessel size as indicated by recommendations (21). Brachial artery FMD was measured above the antecubital fossa. Ultrasound measures were performed by an expert sonographer with >500 hours of experience. Data were synchronized throughout the experiment by the use of markers.

Experimental protocol

The right forearm vasodilation was assessed after the release of circulatory forearm occlusion at rest (without SYMP) and SYMP conditions. After 30 minutes of supine resting, the brachial cuff was inflated for 5 minutes and then released to induce forearm vasodilation. After an additional 30 minutes of supine rest, the same procedure was repeated during SYMP. A previous study suggested that FMD assessment could be performed several times with almost identical results with 30 minutes of rest in between [23]. In accordance with the methodology and timing used in the study by Dyson et al. [19], SYMP was induced by CPT by dipping subjects' feet in an ice-water slurry (5°C). The cuff inflation was initiated 2 minutes after feet submersion in the slurry. The stimulus continued until the conclusion of the experiment. Data were gathered at baseline for 1 minute, during the entire occlusion time (5 minutes), and after cuff release (3 minutes).

Vascular data

The forearm VC was calculated by measuring MAP and BF through the brachial artery. Ultrasound data (video clips) were downloaded from the Ultrasound device and analyzed via software (Medical Imaging Applications LLC, USA). The software provided automatic detection of the artery edges along with the mean blood velocity calculation. The diameter was measured every cardiac cycle at the onset of R-waves. Portapres data were exported through its proprietary software (BeatScope 1.1; Finapres Medical System BV, The Netherlands). Ultrasound data (brachial artery diameter, mean blood velocity) and MAP data were analyzed beat-by-beat with a 3-beat rolling average. Then, data were fitted to avoid erroneous calculations of peak forearm VC or peak brachial artery FMD [19], as well as to extrapolate second-by-second data. Brachial artery BF was calculated as mean blood velocity (cm*s−1)*π*r2*60 (ml*min−1), where r is the radius of the brachial artery. Forearm VC was calculated as brachial artery BF divided by MAP.

The primary research outcome was the percent increase from preceding baseline values of forearm VC upon cuff release. We calculated the percent increase from baseline of peak forearm VC after cuff release as well as the percent increase from baseline of the average forearm VC over the 60 seconds following cuff release. We also calculated the difference of such percent increments in forearm VC between rest and SYMP (delta value: percent increase at rest minus percent increase at SYMP), in order to quantify the extent to which SYMP restrains the normal increase in VC in both sexes. Similar calculations were repeated for brachial artery BF data. The brachial artery FMD was an additional research outcome. Brachial artery peak FMD was also calculated as the percent increase from baseline after cuff release [162, 163]. Shear rate was determined as 4 times mean blood velocity divided by artery diameter. The peak value of shear rate upon cuff release was identified. The cumulative shear rate was calculated as the area under the curve (AUC) from cuff release to the peak brachial artery diameter. Brachial artery FMD was normalized to cumulative shear rate and baseline diameter. Additionally, allometric scaling of FMD was also calculated as previously indicated [165] to control for statistical bias towards the brachial artery baseline diameter.

Hemodynamic data

Beat-by-beat cardiac output, stroke volume, MAP, total peripheral resistance (TPR), and heart rate were non-invasively calculated through the Modelflow algorithm of Portapres. These data were recorded on the left hand to obtain information on systemic changes before, during, and after circulatory occlusion at rest and during SYMP. Hemodynamic data were averaged over the 60 seconds after cuff release, in concomitance with the vascular data collection, in both rest and SYMP conditions. The percent changes from the previous baseline values (pre-cuff inflation) of all hemodynamic data after cuff release were calculated.

NIRS data

The average oxygen saturation of the thenar eminence was calculated at baseline, over the 60 seconds before cuff release, and over the 60 seconds following cuff release. Values of oxygen saturation during ischemia and after cuff release are reported as percent changes from the preceding baseline values.

Statistics

Statistical comparison was performed on the data collected at rest versus during SYMP in men versus women. Data normality was tested with the Shapiro-Wilk normality test. A two-way repeated-measure ANOVA with a Sidak posthoc test was used to assess any effects of SYMP and sex on the forearm VC, brachial artery BF, hemodynamic data, and hand oxygenation. GraphPad Prism 8 (GraphPad Software, San Diego, United States) was used for statistical analysis and graphs. The analysis of covariance required for the allometric scaling of the brachial artery FMD was performed with MATLAB (MathWorks, USA). Results are expressed as mean ± standard deviations.

RESULTS

All data passed the normality test. ANOVA results are reported in Table 2. Numerical data and effect sizes are also provided in the supplementary file.

	Effect of sex	Effect of SYMP	Interaction
Peak forearm VC change (%)	p=0.97	p<0.0001	p=0.42
Mean forearm VC change (%)	p=0.84	p<0.0001	p=0.94
Peak brachial artery BF change (%)	p=0.95	p<0.0001	p=0.25
Mean brachial artery BF change (%)	p=0.73	p=0.03	p=0.54
Hand oxygenation after cuff inflation (%)	p=0.70	p=0.16	p=0.54
Hand oxygenation after cuff release (%)	p=0.004	p=0.009	p=0.80
Brachial artery FMD (%)	p=0.03	p<0.0001	p=0.03
FMD normalized to bsl diameter (% x cm)	p=0.25	p<0.0001	p=0.11
FMD normalized to cumulative shear stress (e^{-005} %/s^{-1}x60s)	p=0.08	p<0.0001	p=0.34
TPR change (%)	p=0.04	p=0.01	p=0.03
MAP change (%)	p=0.41	p<0.0001	p=0.43
Heart rate change (%)	p=0.98	p<0.0001	p=0.83
Cardiac output change (%)	p=0.75	p=0.50	p=0.55
Cardiac stroke volume change (%)	p=0.22	p=0.23	p=0.85

Table 2. The table reports the ANOVA results.

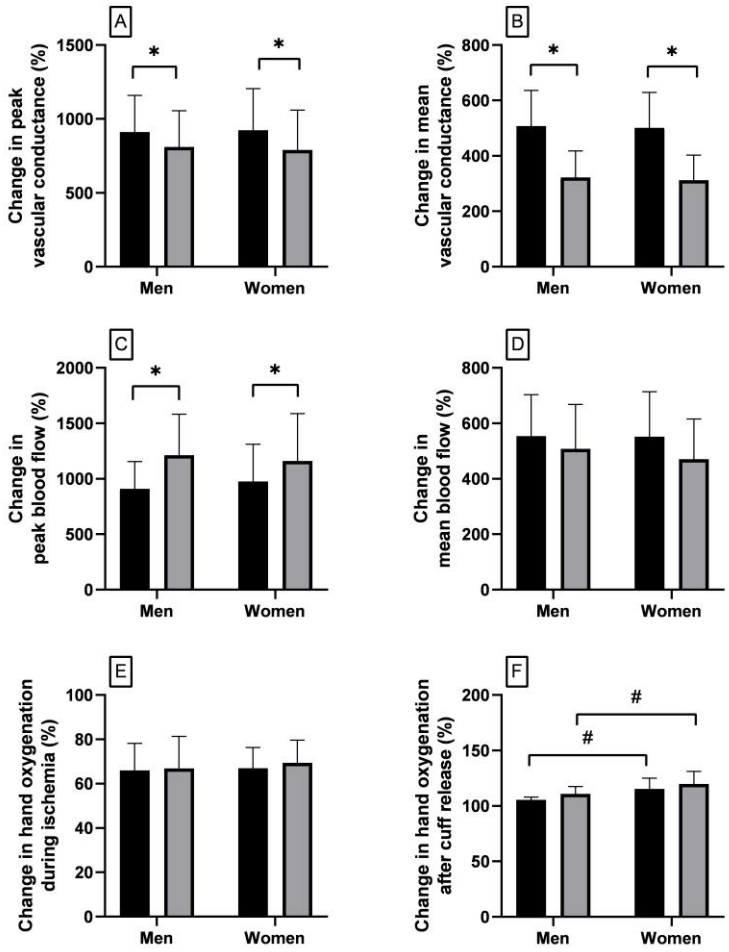

Figure 1. Percent changes from preceding baseline values after cuff release at rest (black bars) versus SYMP (grey bars) ($p<0.05$) conditions in men versus women (# $p<0.05$).*

Vascular conductance

Baseline forearm VC (Table 1) was lower in women compared to men ($p=0.03$). The percent increase from baseline of peak forearm VC after cuff release (Figure 1A) did not differ between sexes either at rest ($p=0.99$) or during SYMP ($p=0.98$) and was blunted by SYMP in both sexes ($p<0.003$). The percent increase from baseline of the average forearm VC over the 60 seconds after cuff release (Figure 1B) did not differ between sexes either at rest ($p=0.98$) or during SYMP ($p=0.97$) and was blunted by SYMP in both sexes ($p<0.0001$). Therefore, SYMP

restrained the peak (Figure 3A; p=0.42) and average (Figure 3B; p=0.94) forearm VC increments to a similar degree in men compared to women. Absolute values of forearm VC after cuff release are reported in the supplementary file.

Blood flow

Baseline brachial artery BF (Table 1) was lower in women compared to men (p=0.01). The percent increase from baseline of peak BF after cuff release (Figure 1C) did not differ between sexes at rest (p=0.87) or during SYMP (p=0.92), but was increased by SYMP in both sexes (men: p=0.0007; women: p=0.03). The percent increase from baseline of the average BF over the 60 seconds after cuff release (Figure 1D) did not differ between sexes at rest (p=0.99) or during SYMP (p=0.80), and was unaffected by SYMP in both sexes (men: p=0.45; women: p=0.09). Therefore, SYMP augmented the normal peak BF (Figure 3C; p=0.25) and restrained the average BF (Figure 3D; p=0.54) to a similar degree in men compared to women. Absolute values of BF after cuff release are reported in the supplementary file. Absolute values of peak BF resulted to be statistically augmented in men (p=0.004) but not in women (p=0.46) during SYMP compared to at rest.

Hand Oxygenation

Compared to the previous baseline values (100%), the values of hand oxygenation reached in the minute prior to the cuff opening (Figure 1E) were similar in men compared to women, both at rest (p=0.97) and during SYMP (p=0.83), and were not affected by SYMP (men: p=0.82; women: p=0.28). The average hand oxygenation over the 60 seconds after cuff release (Figure 1F) reached values above the preceding baseline in both sexes. However, the values of hand oxygenation reached after cuff release were higher in women compared to men both at rest (p=0.01) and during SYMP (p=0.02), whereas they were not affected by SYMP in both sexes (men: p=0.08; women: p=0.14).

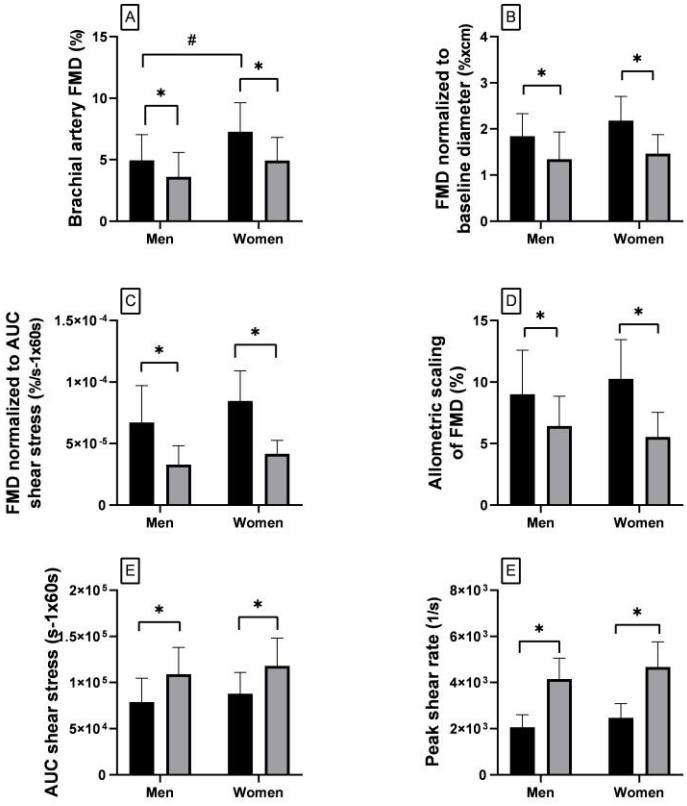

Figure 2. Brachial artery flow-mediated dilation (FMD) changes after cuff release at rest (black bars) versus SYMP (grey bars) ($p<0.05$) conditions in men versus women (# $p<0.05$).*

Brachial artery FMD

Baseline brachial artery diameter (Table 1) was lower in women than in men ($p=0.002$). Prior to performing any normalization, brachial artery FMD (Figure 2A) was higher in women than men at rest ($p=0.016$), whereas it was similar during SYMP ($p=0.23$). FMD normalized to baseline diameter (Figure 2B) at rest ($p=0.20$) and during SYMP ($p=0.80$), and FMD normalized to cumulative shear rate (Figure 2C) at rest ($p=0.09$) and during SYMP ($p=0.53$), were not different between sexes. However, they were affected by SYMP in both sexes (all $p<0.0002$). Therefore, SYMP-mediated blunting of brachial artery FMD was greater in women compared to men prior to performing any normalization (Figure 3E; $p=0.03$), whereas it was no longer different after normalization to baseline brachial artery diameter (Figure

3F; p=0.11) and shear rate (Figure 3G; p=0.34). Allometric scaling of FMD (Figure 2D) also revealed that brachial artery dilation was similar in men compared to women at rest (9.01±3.57% vs 10.25±3.19%, men vs women; p=0.49) and during SYMP (6.43±2.41% vs 5.55±1.98%; p=0.51).

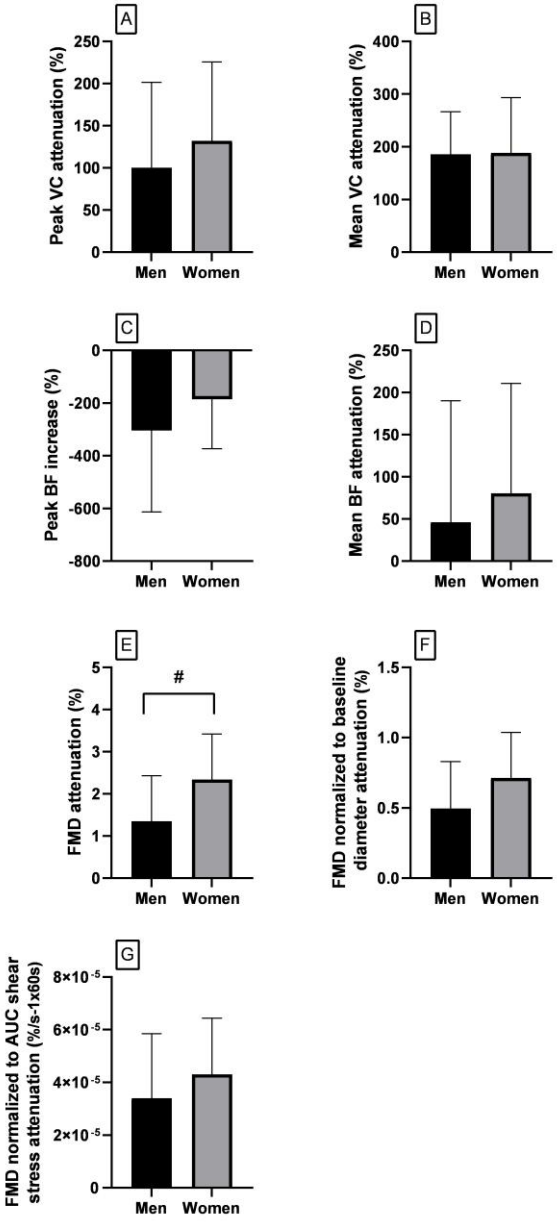

Figure 3. Delta changes (percent increase from baseline at rest minus at SYMP) due to SYMP after cuff release (# p<0.05).

Hemodynamic data

TPR over the 60 seconds after cuff release (Figure 4A) was augmented during SYMP compared to at rest in men ($p=0.004$) but not in women ($p=0.967$). Prior to normalization to baseline values, absolute values of MAP (supplementary file) were similar in women compared to men at rest ($p=0.24$), but lower in women during SYMP ($p=0.005$). When the different baseline MAPs between sexes were accounted for, the percent increase from baseline of MAP (Figure 3B) during SYMP was similar in men compared to women ($p=0.436$). Moreover, MAP increased in response to SYMP in both sexes ($p<0.0001$). The heart rate (Figure 3C) increased in response to SYMP in both sexes ($p<0.009$) and the percent changes from baseline were similar between sexes ($p>0.97$). Cardiac output (Figure 3D) and stroke volume (Figure 3E) were not affected by SYMP in both sexes ($p>0.53$) and the percent changes from baseline were similar between sexes ($p>0.46$).

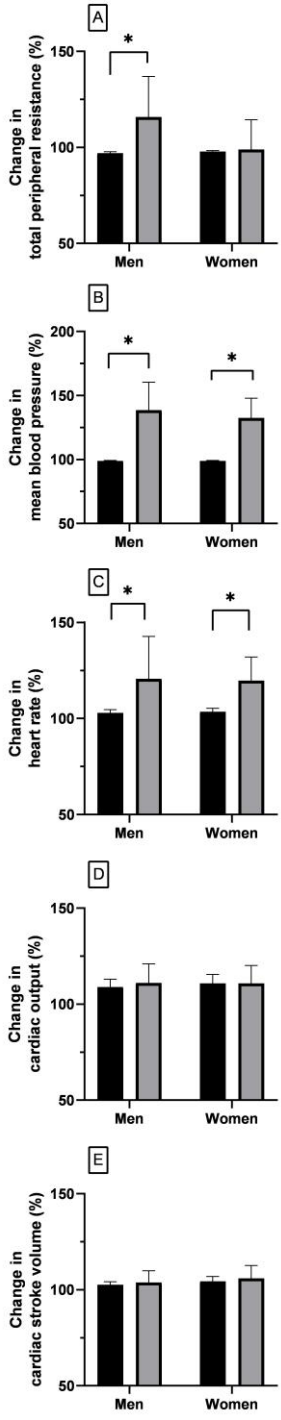

Figure 4. Percent changes from preceding baseline values (pre-cuff inflation) at rest (black bars) versus SYMP (grey bars) ($p<0.05$) conditions in men versus women (# $p<0.05$).*

DISCUSSION

Summary and main findings

This study investigated sex differences in the in-vivo forearm VC regulation in young healthy humans. The in-vivo regulation of VC is a continuous balance between sympathetic vasoconstriction and endothelial-mediated vasodilation [113]. The precise regulation of VC is essential for controlling tissue BF and systemic blood pressure [159]. The interaction between sympathetic vasoconstriction and endothelial-mediated vasodilation was evaluated by assessing the sympathetic-mediated blunting of forearm vasodilation after a brief period of ischemia. It was also assessed whether any sympathetic-mediated restriction of vasodilation blunts tissue BF and re-oxygenation after circulatory reperfusion in both sexes. Our data may agree with previous investigations suggesting that women have blunted transduction of sympathetic activity into vasoconstriction compared to men. Indeed, TPR was augmented in response to CPT-induced SYMP in men but not in women. Although the higher brachial artery FMD found in women compared to men may lead to the initial conclusion that women have greater endothelial-mediated vasodilation, this non-normalized result is biased by the smaller baseline brachial artery diameter in women. Indeed, no sex differences in brachial artery FMD were found after normalization to baseline diameter (details below). The percent increases from baseline of peak and average forearm VC after circulatory occlusion, at rest, were similar between sexes. Interestingly, such increases were also similar between sexes during SYMP, regardless of the fact that women showed a blunted increase in TPR in response to SYMP. Thus, as shown in Figure 3, sympathetic-mediated restriction of vasodilation was similar between sexes. The percent increase from baseline of the average brachial artery BF after cuff release was also similar between sexes at rest and during SYMP. Although SYMP restrained the average forearm VC and BF increments over the 60 seconds after release of circulatory occlusion, such restrictions did not impair the normal hand re-oxygenation that occurs in that time frame in both sexes.

CPT was used to induce SYMP. CPT is a painful stressor that elevates blood pressure mainly due to peripheral sympathetic vasoconstriction [166]. This

stimulant was used in several investigations to assess the sympathetic-mediated blunting of nitric oxide-mediated endothelial vasodilation [19, 23, 167]. The specific timing of application of the CPT stimulus used in our experimental protocol was taken from the study by Dyson et al. [19] in order to have a direct comparison between previous findings and our own. CPT-induced SYMP has been shown to blunt the normal brachial artery vasodilation upon release of circulatory occlusion [19, 23, 167]. Other non-invasive sympathetic stimulants such as mental arithmetic tasks, lower body suction, and muscle chemoreflex activation failed in this intent [19]. The percent increase from baseline of forearm VC following reperfusion was considered the main research outcome in our study. Increases in VC provide information on the global vasodilation of the forearm, including changes in resistance arterioles and pre-capillary arterioles. Restrictions in the normal forearm VC increase after a brief period of ischemia can potentially affect BF towards the hand and, thus, the hand re-oxygenation in that time frame. The brachial artery FMD was a secondary research outcome in our study. It provides information on nitric oxide-mediated endothelial vasodilation, which has been associated with coronary artery endothelial function [168]. However, brachial artery FMD does not provide information on the precise regulation of BF towards the hand [169]. Hemodynamic changes were calculated via the ModelFlow algorithm of Portapres. This algorithm has been widely validated and used in research [170, 171]. It uses a statistical model of the human circulation to calculate the hemodynamic parameters from the finger arterial pressure waveform.

Hemodynamic data

Our data show that TPR after cuff release was augmented in men but not in women during CPT-induced SYMP compared to at rest (Figure 4A). This finding may agree with the results of a previous study that sought to quantify leg sympathetic vasoconstriction in response to CPT in young individuals [164]. CPT induced a blunted decrease in femoral vascular conductance in young women compared to young men, consistent with the notion of a blunted increase in regional

peripheral resistance in women [164]. Other studies also showed that sympathetic stimulants induce less vasoconstriction in women compared to men [122]. A blunted increase in TPR in women may agree with the notion that women have blunted transduction of MSNA into vasoconstriction compared to men [89, 122, 123]. Indeed, a lack of relationship between MSNA and TPR in women but not in men has been documented [122]. Moreover, women have been suggested to have lower autonomic support of arterial blood pressure control compared to men, as women show a lower arterial pressure drop in response to autonomic blockade compared to men [172]. In our study, men reported higher absolute values of MAP during SYMP compared to women. However, men also reported (not statistically significant) higher values of MAP than women at baseline. After normalization to baseline values, SYMP resulted in similar percent increases of MAP between sexes (Figure 4B). These results support the notion that the use of absolute values may lead to misleading conclusions if differences in baseline values between sexes are not accounted for. The heart rate was increased during SYMP compared to at rest in both sexes. This finding is consistent with the positive chronotropic effect of sympathetic stimulation on the heart rate [173]. Cardiac output and stroke volume were not affected by SYMP in both sexes. These findings agree with those of previous research showing that cardiac index (cardiac output normalized to body surface area) was unchanged during CPT although TPR increased [23].

Interaction between vasoconstriction and vasodilation

The precise regulation of VC is essential to regulate BF towards tissues [159]. VC rapidly increases upon release of circulatory occlusion to allow fast blood supply towards the ischemic area. Rapid vasodilation is due to the release of local vasodilator agents produced by the vascular endothelium or muscle itself that quickly relax the vascular smooth muscle [121]. Our data show that the percent increments from baseline of peak (Figure 1A) and average forearm VC (Figure 1B) after cuff release were similar between sexes at rest. Brachial artery FMD was also similar between men and women after normalization to baseline brachial artery diameter and shear rate (Figure 2B; 2C; 2D). Therefore, when baseline values are

accounted for, similar forearm VC increments and brachial artery FMDs after cuff release in men compared to women may suggest similar endothelial-mediated vasodilation between sexes. Although women may have a blunted sympathetic vasoconstriction compared to men in response to CPT-induced SYMP, as evinced by the blunted TPR increase in women, the percent increase from baseline of forearm VC after cuff release was similar between sexes during SYMP. Thus, SYMP blunted the normal VC increments (Figure 3A; 3B) after cuff release similarly between sexes. These findings suggest that, when comparisons are performed on data normalized to preceding baseline values, there are no overt sex differences in the dynamic regulation of forearm VC assessed through the interaction between endothelial-mediated vasodilation and sympathetic vasoconstriction. SYMP blunted the peak and average forearm VC increase upon cuff release in both sexes. No study has specifically investigated whether SYMP restrains the normal increase in forearm VC, however, SYMP-mediated restriction of vasodilation is consistent with the physiological assumptions. Norepinephrine released from sympathetic nerve endings and adrenal glands during SYMP should oppose the normal nitric oxide-mediated vascular smooth muscle relaxation by binding to post-synaptic α-adrenergic receptors [9].

The percent increments from baseline of peak (Figure 1C) and average (Figure 1D) brachial artery BF after cuff release were similar between sexes at rest and during SYMP. Thus, SYMP changed the normal BF responses (Figure 3C; 3D) after cuff release similarly between sexes. These results suggest that, when different baseline values of BF between sexes are accounted for, there are no overt sex differences in the dynamic regulation of brachial artery BF towards an ischemic area regardless of a stressful situation. SYMP augmented the percent increase from baseline of peak brachial artery BF in both sexes. This is probably due to the higher limb perfusion pressure (MAP) during SYMP which overpowers the effects of any restrained vasodilation [159]. When comparing changes in absolute values of BF, without taking into account different baseline values between sexes, results may be misleading. Absolute values of peak BF during SYMP compared to at rest resulted to be statistically augmented in men but not in women. This finding may provide further insight into the study by Lind et al. [23], where brachial artery BF

immediately after cuff release increased without reaching statistical significance during CPT as compared to without sympathetic stress in a group composed of 10 young men and 8 young women. A blunted change in the absolute peak BF in response to SYMP in women might be responsible for the failure to achieve statistical significance in the previous study. SYMP did not change the percent increment from baseline of the average brachial artery BF over the 60 seconds after cuff deflation. This suggests that acute levels of sympathetic activation do not impair the normal capacity to provide blood towards an ischemic tissue despite the presence of a blunted forearm vasodilation in both sexes, probably due to a weighted increase of MAP [159].

Hand oxygenation diminished to similar levels after 5 minutes of forearm cuff occlusion in both sexes, regardless of SYMP (Figure 1E). Hand oxygenation then increased above preceding baseline levels in both sexes over the 60 seconds after cuff release, without being impaired by SYMP (Figure 1F). This suggests that SYMP does not impair the normal hand re-oxygenation after cuff release despite it blunts forearm vasodilation in both sexes. Similar values of hand oxygenation over the 60 seconds after cuff release during SYMP compared to at rest may be explained by the fact that SYMP does not change the average hyperemic response in that time frame. However, the values of hand oxygenation reached after cuff release were greater in women compared to men. Since the average brachial artery BF after cuff release was similar between sexes, a faster peripheral oxygen extraction dynamic in women compared to men might be involved [174].

Consistent with previous investigations, brachial artery FMD from baseline was higher in women compared to men when expressed as absolute values (Figure 2A) [158, 175]. However, as previously shown, sex differences were abolished after normalization to baseline diameter [175] and cumulative shear rate (Figure 2B; 2C; 2D). Women generally have a smaller brachial artery diameter compared to men [158, 175]. The baseline artery diameter has been shown to affect artery FMD, as FMD is higher in smaller arteries due to the higher shear rate during reactive hyperemia, and vice versa [158, 163]. In our study, SYMP blunted the brachial artery FMD in both sexes. This is in agreement with previous research [19, 23] which, however, did not investigate sex differences. In this regard, our data show

that FMD attenuation by SYMP was higher in women compared to men when expressed as absolute values (Figure 3E). Previous studies suggested a sex-related sensitivity in the regulation of large-artery vascular tone, as evinced by more pronounced shear-mediated arterial vasodilation and vasoconstriction in women compared to men [176]. Consistent with this notion, sex differences in brachial artery FMD attenuation were abolished after normalization to shear rate and baseline diameter (Figure 3F; 3G).

Limitations

This study did not aim to provide the physiological mechanisms underlying sex differences. As previously done in similar studies [167, 177], we did not measure MSNA, cortisol, epinephrine, norepinephrine, or oestrogen. Evaluation of these variables could provide further clarification regarding sex differences in neurovascular modulation. Using the analogous experimental protocol employed in our project, other researchers showed that CPT increases only blood norepinephrine by 1 minute after cuff deflation in young men, without affecting blood epinephrine and serum cortisol [19]. Other studies, however, have suggested that some variables change immediately after the stress application, whereas others show delayed responses such as peak cortisol concentration [177].

Perspectives and Significance

Neurovascular regulation is based on the continuous interaction between sympathetic vasoconstriction and endothelial-mediated vasodilation. However, the current understanding of sex differences in neurovascular regulation is predominantly based on studies in which these two aspects have been investigated separately. The lack of knowledge of how these two aspects interact could lead to apparently rational, but erroneous, speculations about neurovascular regulation in-vivo, such as the hypothesis we tested that men had greater sympathetic blunting of endothelial-mediated vasodilation. Our data normalized to baseline values show similar sympathetic-mediated restriction of vasodilation between sexes. This

suggests that speculations on how the dynamic neurovascular regulation differs between sexes cannot be based upon "mathematical operations" between vasodilation and vasoconstriction differences assessed separately. Furthermore, previous similar investigations have focused on the sympathetic-mediated blunting of FMD of the brachial artery. This variable is related to cardiovascular risk but is not a key regulator of brachial artery BF and blood pressure. Therefore, the results of our study encourage investigating sex differences in the interaction between sympathetic vasoconstriction and endothelial-mediated vasodilation on VC, as well as how this integrated regulation eventually affects tissue BF and blood pressure.

Conclusion

When considered separately, women show similar endothelial vasodilation compared to men, as well as a likely blunted sympathetic vasoconstriction in response to CPT-induced SYMP. However, the interaction between vasoconstriction and vasodilation leads to a similar regulation of forearm VC between sexes. Indeed, sympathetic-mediated restriction of vasodilation is similar between sexes. Although SYMP restrains the normal forearm VC increase in both sexes, SYMP does not impair the normal hyperemic response or the normal hand re-oxygenation after a brief period of ischemia in both sexes.

APPENDIX 3: MANUSCRIPTS AUTHORED DURING THE PHD PROGRAM

Published manuscripts

- Gentilin A, Moghetti P, Cevese A, Mattioli AV, Schena F, Tarperi C. Circadian and sex differences in carotid-femoral pulse wave velocity in young individuals and elderly with and without type 2 diabetes. Frontiers in Cardiovascular Medicine – in press
- Gentilin A, Moghetti P, Cevese A, Mattioli AV, Schena F, Tarperi C. Circadian variations in sympathetic vasoconstriction in older adults with and without type 2 diabetes. High Blood Press. Cardiovasc. Prev. 2023 – in press
- Gentilin A, Cevese A, Schena F, Tarperi C. Mental stress-induced sympathetic activation augments central artery stiffness in young individuals of both sexes. Biol. Psychol. 2023 – in press
- Gentilin A, Moghetti P, Cevese A, Schena F, Tarperi C. Sympathetic-mediated blunting of forearm vasodilation is similar between young men and women. Biol Sex Differ. 2022 Jun 25;13(1):33.
- Gentilin A, Tarperi C, Cevese A, Mattioli AV, Schena F. Estimation of carotid-femoral pulse wave velocity from finger photoplethysmography signal. Physiol Meas. 2022 Jul 18;43(7).
- Gentilin A, Tarperi C, Skroce K, Cevese A, Schena F. Effects of acute sympathetic activation on the central artery stiffness after strenuous endurance exercise. Sport Sci Health 2022.
- Gentilin A, Zanini P, Cevese A, Schena F, Tarperi C. Ergogenic effects of citrulline supplementation on exercise performance and physiological indexes of exercise performance during cycling tests: a review. Sci and sports– in press
- Gentilin A, Tarperi C, Skroce K, Cevese A, Schena F. Effect of acute sympathetic activation on leg vasodilation before and after endurance exercise. J Smooth Muscle Res. 2021;57(0):53-67.

- Gentilin A, Budel L, Cevese A, Schena F, Tarperi C. Uphill vs downhill high-intensity training in preserving vasodilation capacity and exercise performance. Sport Sci Health 2022 – in press
- Gentilin A, Tam E, Tarperi C, Cevese A, Schena F. Post-exercise upside-down recovery does accelerate the heart rate recovery but does not improve subsequent sprint performance. J Sports Med Phys Fitness. 2021
- Gentilin A, Tecchio P, Cevese A, Schena F, Tarperi C. Pedaling cadence variability increases with worsened bike fitting across most, but not all workloads. Mov Sport Sci – in press

Under revision manuscripts as of 16/03/2023

- Gentilin A, Moghetti P, Cevese A, Mattioli AV, Schena F, Tarperi C. Circadian and sex differences in post-ischemic vasodilation and reactive hyperemia in young individuals and elderly with and without type 2 diabetes.

BIBLIOGRAPHY

1. Wehrwein EA, Orer HS, Barman SM (2016) Overview of the Anatomy, Physiology, and Pharmacology of the Autonomic Nervous System. In: Comprehensive Physiology. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp 1239–1278

2. McCorry LK (2007) Physiology of the autonomic nervous system. Am J Pharm Educ 71:78

3. Mitchell JH, Victor RG (1996) Neural control of the cardiovascular system: insights from muscle sympathetic nerve recordings in humans. Med Sci Sports Exerc 28:S60-9

4. Grubb BP, Kosinski DJ, Kanjwal Y (2002) Neurovegetative Regulation of the Vascular System. In: Pan Vascular Medicine. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 175–187

5. Hasan W (2013) Autonomic cardiac innervation: development and adult plasticity. Organogenesis 9:176–93. https://doi.org/10.4161/org.24892

6. Higgins CB, Vatner SF, Braunwald E (1973) Parasympathetic Control of the Heart. Pharmacol Rev 25:

7. Buckwalter JB, Clifford PS (2001) The paradox of sympathetic vasoconstriction in exercising skeletal muscle. Exerc Sport Sci Rev 29:159–163. https://doi.org/10.1097/00003677-200110000-00005

8. Silber HA, Ouyang P, Bluemke DA, et al (2005) Why is flow-mediated dilation dependent on arterial size? Assessment of the shear stimulus using phase-contrast magnetic resonance imaging. Am J Physiol - Hear Circ Physiol 288:. https://doi.org/10.1152/ajpheart.00612.2004

9. Shoemaker JK, Badrov MB, Al-Khazraji BK, Jackson DN (2016) Neural control of vascular function in skeletal muscle. Compr Physiol 6:303–329. https://doi.org/10.1002/cphy.c150004

10. Cohen RA, Weisbrod RM, Gericke M, et al (1999) Mechanism of nitric oxide-induced vasodilatation: Refilling of intracellular stores by

sarcoplasmic reticulum Ca2+ ATPase and inhibition of store-operated Ca2+ influx. Circ Res 84:210–219. https://doi.org/10.1161/01.RES.84.2.210

11. Clifford PS, Hellsten Y (2004) Vasodilatory mechanisms in contracting skeletal muscle. J Appl Physiol 97:393–403. https://doi.org/10.1152/japplphysiol.00179.2004

12. Marshall J (2007) The roles of adenosine and related substances in exercise hyperaemia. J Physiol 583:835. https://doi.org/10.1113/JPHYSIOL.2007.136416

13. Jackson WF (2017) Potassium Channels in Regulation of Vascular Smooth Muscle Contraction and Growth. Adv Pharmacol 78:89. https://doi.org/10.1016/BS.APHA.2016.07.001

14. Green D, Hopkins N, Jones H, et al (2016) Sex differences in vascular endothelial function and health in humans: impacts of exercise. Exp Physiol 101:230–242. https://doi.org/10.1113/EP085367

15. Stanhewicz A, Wenner M, Stachenfeld N (2018) Sex differences in endothelial function important to vascular health and overall cardiovascular disease risk across the lifespan. Am J Physiol Heart Circ Physiol 315:H1569–H1588. https://doi.org/10.1152/AJPHEART.00396.2018

16. Hashimoto M, Akishita M, Eto M, et al (1995) Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation 92:3431–3435. https://doi.org/10.1161/01.CIR.92.12.3431

17. Jentsch VL, Wolf OT (2020) The impact of emotion regulation on cardiovascular, neuroendocrine and psychological stress responses. Biol Psychol 154:. https://doi.org/10.1016/J.BIOPSYCHO.2020.107893

18. Middlekauff HR, Nguyen AH, Negrao CE, et al (1997) Impact of acute mental stress on sympathetic nerve activity and regional blood flow in advanced heart failure: implications for “triggering” adverse cardiac events.

Circulation 96:1835–1842. https://doi.org/10.1161/01.CIR.96.6.1835

19. Dyson KS, Shoemaker JK, Hughson RL (2006) Effect of acute sympathetic nervous system activation on flow-mediated dilation of brachial artery. Am J Physiol - Hear Circ Physiol 290:H1446-53. https://doi.org/10.1152/ajpheart.00771.2005

20. Bylsma LM, Yaroslavsky I, Rottenberg J, et al (2015) Juvenile onset depression alters cardiac autonomic balance in response to psychological and physical challenges. Biol Psychol 110:167. https://doi.org/10.1016/J.BIOPSYCHO.2015.07.003

21. Matthews KA, Woodall KL, Allen MT (1993) Cardiovascular reactivity to stress predicts future blood pressure status. Hypertens (Dallas, Tex 1979) 22:479–485. https://doi.org/10.1161/01.HYP.22.4.479

22. Matthews KA, Salomon K, Brady SS, Allen MT (2003) Cardiovascular reactivity to stress predicts future blood pressure in adolescence. Psychosom Med 65:410–415. https://doi.org/10.1097/01.PSY.0000057612.94797.5F

23. Lind L, Johansson K, Hall J (2002) The effects of mental stress and the cold pressure test on flow-mediated vasodilation. Blood Press 11:22–27. https://doi.org/10.1080/080370502753543927

24. Pernow J, Schwieler J, Kahan T, et al (1989) Influence of sympathetic discharge pattern on norepinephrine and neuropeptide Y release. Am J Physiol 257:. https://doi.org/10.1152/AJPHEART.1989.257.3.H866

25. Johnson CD, Coney AM, Marshall JM (2001) Roles of norepinephrine and ATP in sympathetically evoked vasoconstriction in rat tail and hindlimb in vivo. Am J Physiol Heart Circ Physiol 281:. https://doi.org/10.1152/AJPHEART.2001.281.6.H2432

26. Hijmering ML, Stroes ESG, Olijhoek J, et al (2002) Sympathetic activation markedly reduces endothelium-dependent, flow-mediated vasodilation. J Am Coll Cardiol 39:683–688. https://doi.org/10.1016/S0735-

1097(01)01786-7

27. Güney HZ, Hodoğlugil U, Uluoğlu C, et al (1998) In vitro susceptibility rhythms. II. Biological-time-dependent differences in effect of β1 and β2-adrenergic agonists of rat aorta and influence of endothelium. Chronobiol Int 15:159–172. https://doi.org/10.3109/07420529808998680

28. Otto ME, Svatikova A, De Mattos Barretto RB, et al (2004) Early morning attenuation of endothelial function in healthy humans. Circulation 109:2507–2510. https://doi.org/10.1161/01.CIR.0000128207.26863.C4

29. Chen L, Yang G (2015) Recent advances in circadian rhythms in cardiovascular system. Front. Pharmacol. 6:71

30. Morris CJ, Yang JN, Scheer FAJL (2012) The impact of the circadian timing system on cardiovascular and metabolic function. Prog Brain Res 199:337–358. https://doi.org/10.1016/B978-0-444-59427-3.00019-8

31. Zhao X, Guan J (2018) Autonomic nervous system might be related with circadian rhythms and have the intricate effects in obstructive sleep apnea with metabolic syndrome. J Clin Hypertens 20:1553–1553. https://doi.org/10.1111/jch.13378

32. Scheer FAJL, Hu K, Evoniuk H, et al (2010) Impact of the human circadian system, exercise, and their interaction on cardiovascular function. Proc Natl Acad Sci U S A 107:20541–20546. https://doi.org/10.1073/pnas.1006749107

33. Scheer FAJL, Hilton MF, Mantzoros CS, Shea SA (2009) Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci U S A 106:4453–4458. https://doi.org/10.1073/pnas.0808180106

34. Ruger M, Scheer FAJL (2009) Effects of circadian disruption on the cardiometabolic system. Rev. Endocr. Metab. Disord. 10:245–260

35. Chang AM, Scheer FAJL, Czeisler CA (2011) The human circadian system adapts to prior photic history. J Physiol 589:1095–1102.

https://doi.org/10.1113/jphysiol.2010.201194

36. Takeda N, Maemura K (2010) Circadian clock and vascular disease. Hypertens. Res. 33:645–651

37. Cook RF, Bussey CT, Fomison-Nurse IC, et al (2019) β2-Adrenoceptors indirectly support impaired β1-adrenoceptor responsiveness in the isolated type 2 diabetic rat heart. Exp Physiol 104:808–818. https://doi.org/10.1113/EP087437

38. Chen L, Yang G (2015) Recent advances in circadian rhythms in cardiovascular system. Front. Pharmacol. 6:71

39. Linsell CR, Lightman SL, Mullen PE, et al (1985) Circadian rhythms of epinephrine and norepinephrine in man. J Clin Endocrinol Metab 60:1210–1215. https://doi.org/10.1210/JCEM-60-6-1210

40. Chen L, Yang G (2015) Recent advances in circadian rhythms in cardiovascular system. Front Pharmacol 6:71. https://doi.org/10.3389/FPHAR.2015.00071

41. Elliott WJ (2001) Cyclic and circadian variations in cardiovascular events. Am J Hypertens 14:. https://doi.org/10.1016/S0895-7061(01)02174-4

42. Muller JE, Tofler GH, Verrier RL (1995) Sympathetic activity as the cause of the morning increase in cardiac events. A likely culprit, but the evidence remains circumstantial. Circulation 91:2508–2509. https://doi.org/10.1161/01.CIR.91.10.2508

43. Middlekauff HR, Sontz EM (1995) Morning sympathetic nerve activity is not increased in humans. Implications for mechanisms underlying the circadian pattern of cardiac risk. Circulation 91:2549–2555. https://doi.org/10.1161/01.CIR.91.10.2549

44. Panza JA, Epstein SE, Quyyumi AA (1991) Circadian variation in vascular tone and its relation to alpha-sympathetic vasoconstrictor activity. N Engl J Med 325:986–990. https://doi.org/10.1056/NEJM199110033251402

45. Otto ME, Svatikova A, De Mattos Barretto RB, et al (2004) Early morning attenuation of endothelial function in healthy humans. Circulation 109:2507–2510. https://doi.org/10.1161/01.CIR.0000128207.26863.C4

46. Somers VK, Dyken ME, Mark AL, Abboud FM (1993) Sympathetic-nerve activity during sleep in normal subjects. N Engl J Med 328:303–307. https://doi.org/10.1056/NEJM199302043280502

47. Kollias GE, Stamatelopoulos KS, Papaioannou TG, et al (2009) Diurnal variation of endothelial function and arterial stiffness in hypertension. J Hum Hypertens 23:597–604. https://doi.org/10.1038/JHH.2009.2

48. Sardeli AV, Chacon-Mikahil MPT (2016) Is The Exercise-Induced Increase in Central Arterial Stiffness a Risk Factor for Health? J Arch Mil Med 4:e36833. https://doi.org/10.5812/jamm.36833

49. Seals DR, Jablonski KL, Donato AJ (2011) Aging and vascular endothelial function in humans. Clin. Sci. 120:357–375

50. Herrera MD, Mingorance C, Rodríguez-Rodríguez R, Alvarez de Sotomayor M (2010) Endothelial dysfunction and aging: An update. Ageing Res. Rev. 9:142–152

51. Toda N (2012) Age-related changes in endothelial function and blood flow regulation. Pharmacol. Ther. 133:159–176

52. Joyner MJ, Barnes JN, Hart EC, et al (2015) Neural Control of the Circulation: How Sex and Age Differences Interact in Humans. Compr Physiol 5:193. https://doi.org/10.1002/CPHY.C140005

53. Gentilin A, Tarperi C, Skroce K, et al (2021) Effect of acute sympathetic activation on leg vasodilation before and after endurance exercise. J Smooth Muscle Res 57:53–67. https://doi.org/10.1540/JSMR.57.

54. Ferrara N, Komici K, Corbi G, et al (2014) β-adrenergic receptor responsiveness in aging heart and clinical implications. Front Physiol 4:396. https://doi.org/10.3389/fphys.2013.00396

55. Wu H, Norton V, Cui K, et al (2022) Diabetes and Its Cardiovascular Complications: Comprehensive Network and Systematic Analyses. Front Cardiovasc Med 9:841928. https://doi.org/10.3389/FCVM.2022.841928

56. Schiavoni M, Cosentino F, Camici GG, Luescher TF (2007) Diabetes and endothelial dysfunction: What's the culprit? High Blood Press Cardiovasc Prev 14:5–10. https://doi.org/10.2165/00151642-200714010-00002/FIGURES/3

57. Heesch CM, Abboud FM, Thames MD (1984) Acute resetting of carotid sinus baroreceptors. II. Possible involvement of electrogenic Na+ pump. Am J Physiol - Hear Circ Physiol 16:. https://doi.org/10.1152/ajpheart.1984.247.5.h833

58. Bernardi L (2000) Clinical evaluation of arterial baroreflex activity in diabetes. Diabetes Nutr Metab 13:331–40

59. Emdin M (2001) Autonomic nervous system in diabetes. Ital Hear J Suppl 2:857–862

60. Thaung HPA, Baldi JC, Wang HY, et al (2015) Increased efferent cardiac sympathetic nerve activity and defective intrinsic heart rate regulation in type 2 diabetes. Diabetes 64:2944–2956. https://doi.org/10.2337/db14-0955

61. Muscelli E, Emdin M, Natali A, et al (1998) Autonomic and Hemodynamic Responses to Insulin in Lean and Obese Humans. J Clin Endocrinol Metab 83:2084–2090. https://doi.org/10.1210/jcem.83.6.4878

62. Berne C, Fagius J, Pollare T, Hjemdahl P (1992) The sympathetic response to euglycaemic hyperinsulinaemia - Evidence from microelectrode nerve recordings in healthy subjects. Diabetologia 35:873–879. https://doi.org/10.1007/BF00399935

63. Wang Y, Jiang W, Chen H, et al (2021) Sympathetic Nervous System Mediates Cardiac Remodeling After Myocardial Infarction in a Circadian Disruption Model. Front Cardiovasc Med 8:668387. https://doi.org/10.3389/FCVM.2021.668387

64. Gentilin A, Moghetti P, Cevese A, et al (2022) Circadian and sex differences in carotid-femoral pulse wave velocity in young individuals and elderly with and without type 2 diabetes. Front Cardiovasc Med 9:. https://doi.org/10.3389/FCVM.2022.952621

65. Young BE, Holwerda SW, Vranish JR, et al (2019) Sympathetic Transduction in Type 2 Diabetes Mellitus. Hypertens (Dallas, Tex 1979) 74:201–207. https://doi.org/10.1161/HYPERTENSIONAHA.119.12928

66. Taegtmeyer H, McNulty P, Young ME (2002) Adaptation and maladaptation of the heart in diabetes: Part I. General concepts. Circulation 105:1727–1733. https://doi.org/10.1161/01.CIR.0000012466.50373.E8

67. Soltysinska E, Speerschneider T, Winther S V., Thomsen MB (2014) Sinoatrial node dysfunction induces cardiac arrhythmias in diabetic mice. Cardiovasc Diabetol 13:. https://doi.org/10.1186/s12933-014-0122-y

68. Schofield I, Malik R, Izzard A, et al (2002) Vascular structural and functional changes in type 2 diabetes mellitus: Evidence for the roles of abnormal myogenic responsiveness and dyslipidemia. Circulation 106:3037–3043. https://doi.org/10.1161/01.CIR.0000041432.80615.A5

69. Blum A, Socea D, Sirchan R (2016) Vascular responsiveness in type 2 diabetes mellitus (T2DM). QJM 109:791–796. https://doi.org/10.1093/qjmed/hcw081

70. Donnan PT, Boyle DIR, Broomhall J, et al (2002) Prognosis following first acute myocardial infarction in type 2 diabetes: A comparative population study. Diabet Med 19:448–455. https://doi.org/10.1046/j.1464-5491.2002.00711.x

71. Kanth R, Ittaman S, Rezkalla S (2013) Circadian patterns of ST elevation myocardial infarction in the new millennium. Clin. Med. Res. 11:66–72

72. Vinik AI, Casellini C, Parson HK, et al (2018) Cardiac autonomic neuropathy in diabetes: A predictor of cardiometabolic events. Front Neurosci 12:591. https://doi.org/10.3389/fnins.2018.00591

73. Javeed N, Matveyenko A V. (2018) Circadian etiology of type 2 diabetes mellitus. Physiology 33:138–150. https://doi.org/10.1152/physiol.00003.2018

74. Kurose T, Yabe D, Inagaki N (2011) Circadian rhythms and diabetes. J. Diabetes Investig. 2:176–177

75. Rudic RD, Fulton DJ (2009) Pressed for time: The circadian clock and hypertension. J Appl Physiol 107:1328–1338. https://doi.org/10.1152/japplphysiol.00661.2009

76. Thosar SS, Butler MP, Shea SA (2018) Role of the circadian system in cardiovascular disease. J. Clin. Invest. 128:2157–2167

77. Pothineni NV, Shirazi LF, Mehta JL (2016) Gender Differences in Autonomic Control of the Cardiovascular System. Curr Pharm Des 22:3829–3834

78. Botek M, Krejčí J, McKune A (2018) Sex Differences in Autonomic Cardiac Control and Oxygen Saturation Response to Short-Term Normobaric Hypoxia and Following Recovery: Effect of Aerobic Fitness. Front Endocrinol (Lausanne) 9:697. https://doi.org/10.3389/fendo.2018.00697

79. Dart AM, Du XJ, Kingwell BA (2002) Gender, sex hormones and autonomic nervous control of the cardiovascular system. Cardiovasc. Res. 53:678–687

80. Huikuri H V., Pikkuja¨msa¨ SM, Airaksinen KEJ, et al (1996) Sex-Related Differences in Autonomic Modulation of Heart Rate in Middle-aged Subjects. Circulation 94:122–125. https://doi.org/10.1161/01.CIR.94.2.122

81. Vogel B, Acevedo M, Appelman Y, et al (2021) The Lancet women and cardiovascular disease Commission: reducing the global burden by 2030. Lancet (London, England) 397:2385–2438. https://doi.org/10.1016/S0140-6736(21)00684-X

82. Elder P, Sharma G, Gulati M, Michos ED (2020) Identification of female-

specific risk enhancers throughout the lifespan of women to improve cardiovascular disease prevention. Am J Prev Cardiol 2:100028. https://doi.org/10.1016/J.AJPC.2020.100028

83. Fan W, Song Y, Inzucchi SE, et al (2019) Composite cardiovascular risk factor target achievement and its predictors in US adults with diabetes: The Diabetes Collaborative Registry. Diabetes, Obes Metab 21:1121–1127. https://doi.org/10.1111/dom.13625

84. Regensteiner JG, Golden S, Anton B, et al (2015) Sex differences in the cardiovascular consequences of diabetes mellitus a scientific statement from the American Heart Association. Circulation 132:2424–2447. https://doi.org/10.1161/CIR.0000000000000343

85. Rich-Edwards JW, Kaiser UB, Chen GL, et al (2018) Sex and gender differences research design for basic, clinical, and population studies: Essentials for investigators. Endocr. Rev. 39:424–439

86. Gentilin A, Moghetti P, Cevese A, et al (2022) Sympathetic-mediated blunting of forearm vasodilation is similar between young men and women. Biol Sex Differ 13:33. https://doi.org/10.1186/S13293-022-00444-0

87. Miller VM (2014) Why are sex and gender important to basic physiology and translational and individualized medicine? Am. J. Physiol. - Hear. Circ. Physiol. 306

88. Regitz-Zagrosek V, Kararigas G (2017) Mechanistic Pathways of Sex Differences in Cardiovascular Disease. Physiol Rev 97:1–37. https://doi.org/10.1152/PHYSREV.00021.2015

89. Hissen S, Taylor C (2020) Sex differences in vascular transduction of sympathetic nerve activity. Clin Auton Res 30:381–392. https://doi.org/10.1007/S10286-020-00722-0

90. Vlachopoulos C, Aznaouridis K, Stefanadis C (2010) Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol 55:1318–1327.

https://doi.org/10.1016/J.JACC.2009.10.061

91. Laurent S, Katsahian S, Fassot C, et al (2003) Aortic stiffness is an independent predictor of fatal stroke in essential hypertension. Stroke 34:1203–1206. https://doi.org/10.1161/01.STR.0000065428.03209.64

92. Li Y, Cordes M, Recio-Rodriguez JI, et al (2014) Diurnal variation of arterial stiffness in healthy individuals of different ages and patients with heart disease. Scand J Clin Lab Invest 74:155–162. https://doi.org/10.3109/00365513.2013.864787

93. Dhaun N, Moorhouse R, MacIntyre IM, et al (2014) Diurnal variation in blood pressure and arterial stiffness in chronic kidney disease: the role of endothelin-1. Hypertension 64:296–304. https://doi.org/10.1161/HYPERTENSIONAHA.114.03533

94. Cosentino F, Grant PJ, Aboyans V, et al (2020) 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD. Eur Heart J 41:255–323. https://doi.org/10.1093/EURHEARTJ/EHZ486

95. Van Bortel LM, Laurent S, Boutouyrie P, et al (2012) Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. J Hypertens 30:445–448. https://doi.org/10.1097/HJH.0B013E32834FA8B0

96. Gentilin A, Tarperi C, Cevese A, et al (2022) Estimation of carotid-femoral pulse wave velocity from finger photoplethysmography signal. Physiol Meas 43:075011. https://doi.org/10.1088/1361-6579/AC7A8E

97. Calabia J, Torguet P, Garcia M, et al (2011) Doppler ultrasound in the measurement of pulse wave velocity: Agreement with the Complior method. Cardiovasc Ultrasound 9:13. https://doi.org/10.1186/1476-7120-9-13

98. Saito M, Mano T, Abe H, Iwase S (1986) Responses in muscle sympathetic nerve activity to sustained hand-grips of different tensions in humans. Eur J

Appl Physiol Occup Physiol 55:493–498. https://doi.org/10.1007/BF00421643

99. Mäki-Petäjä KM, Barrett SML, Evans S V., et al (2016) The role of the autonomic nervous system in the regulation of aortic stiffness. Hypertension 68:1290–1297. https://doi.org/10.1161/HYPERTENSIONAHA.116.08035

100. Gentilin A, Tarperi C, Skroce K, et al (2022) Effects of acute sympathetic activation on the central artery stiffness after strenuous endurance exercise. Sport Sci Health 1:1–9. https://doi.org/10.1007/S11332-022-00941-0/FIGURES/2

101. Acanfora D, Casucci G, Ciccone MM, et al (2020) Biomechanical and neuroautonomic adaptation to acute blood volume displacement in ischemic dilated cardiomyopathy: the predictive value of the CD25 test. J Appl Physiol 129:1173–1182. https://doi.org/10.1152/JAPPLPHYSIOL.00514.2019

102. Tan I, Spronck B, Kiat H, et al (2016) Heart Rate Dependency of Large Artery Stiffness. Hypertension 68:236–242. https://doi.org/10.1161/HYPERTENSIONAHA.116.07462

103. DuPont JJ, Kenney RM, Patel AR, Jaffe IZ (2019) Sex differences in mechanisms of arterial stiffness. Br J Pharmacol 176:4208. https://doi.org/10.1111/BPH.14624

104. De Angelis L, Millasseau SC, Smith A, et al (2004) Sex differences in age-related stiffening of the aorta in subjects with type 2 diabetes. Hypertension 44:67–71. https://doi.org/10.1161/01.HYP.0000130482.81883.FD

105. Ji H, Kim A, Ebinger JE, et al (2020) Sex Differences in Blood Pressure Trajectories Over the Life Course. JAMA Cardiol 5:255–262. https://doi.org/10.1001/JAMACARDIO.2019.5306

106. Atkinson G, Jones H, Ainslie PN (2010) Circadian variation in the circulatory responses to exercise: Relevance to the morning peaks in

strokes and cardiac events. Eur J Appl Physiol 108:15–29. https://doi.org/10.1007/s00421-009-1243-y

107. Del Pinto R, Grassi G, Ferri C, et al (2021) Diagnostic and Therapeutic Approach to Sleep Disorders, High Blood Pressure and Cardiovascular Diseases: A Consensus Document by the Italian Society of Hypertension (SIIA). High Blood Press Cardiovasc Prev 28:85–102. https://doi.org/10.1007/S40292-021-00436-Y

108. Thomas GD, Segal SS (2004) Neural control of muscle blood flow during exercise. J Appl Physiol 97:731–738. https://doi.org/10.1152/japplphysiol.00076.2004

109. Michikami D, Kamiya A, Fu Q, et al (2002) Forearm elevation augments sympathetic activation during handgrip exercise in humans. Clin Sci 103:295–301. https://doi.org/10.1042/cs1030295

110. Saito M, Mano T, Iwase S (1990) Changes in muscle sympathetic nerve activity and calf blood flow during static handgrip exercise. Eur J Appl Physiol Occup Physiol 60:277–281. https://doi.org/10.1007/BF00379396

111. Kaijser L (1991) Neurogenic forearm vasodilatation during contralateral isometric exercise is attenuated in diabetes mellitus. Clin Auton Res 1:239–242. https://doi.org/10.1007/BF01824993

112. Jacobsen TN, Hansen J, Nielsen H V., et al (1994) Skeletal muscle vascular responses in human limbs to isometric handgrip. Eur J Appl Physiol Occup Physiol 69:147–153. https://doi.org/10.1007/BF00609407

113. Buckwalter JB, Clifford PS (2001) The paradox of sympathetic vasoconstriction in exercising skeletal muscle. Exerc Sport Sci Rev 29:159–163. https://doi.org/10.1097/00003677-200110000-00005

114. Hamburg NM, Keyes MJ, Larson MG, et al (2008) Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. Circulation 117:2467–2474. https://doi.org/10.1161/CIRCULATIONAHA.107.748574

115. Huang AL, Silver AE, Shvenke E, et al (2007) Predictive Value of Reactive Hyperemia for Cardiovascular Events in Patients With Peripheral Arterial Disease Undergoing Vascular Surgery. Arterioscler Thromb Vasc Biol 27:2113. https://doi.org/10.1161/ATVBAHA.107.147322

116. Philpott A, Anderson TJ (2007) Reactive hyperemia and cardiovascular risk. Arterioscler Thromb Vasc Biol 27:2065–2067. https://doi.org/10.1161/ATVBAHA.107.149740

117. Matteucci E, Giampietro O (2012) Circadian rhythm of blood pressure in diabetes mellitus: evidence, mechanisms and implications. Curr Diabetes Rev 8:355–361. https://doi.org/10.2174/157339912802083496

118. Norhammar A, Schenck-Gustafsson K (2013) Type 2 diabetes and cardiovascular disease in women. Diabetologia 56:1–9. https://doi.org/10.1007/S00125-012-2694-Y

119. Wilkinson IB, McEniery CM, Schillaci G, et al (2010) ARTERY Society guidelines for validation of non-invasive haemodynamic measurement devices: Part 1, arterial pulse wave velocity. Artery Res 4:34–40. https://doi.org/10.1016/j.artres.2010.03.001

120. Mattioli AV (2007) Effects of caffeine and coffee consumption on cardiovascular disease and risk factors. Future Cardiol 3:203–212. https://doi.org/10.2217/14796678.3.2.203

121. Wunsch SA, Muller-Delp J, Delp MD (2000) Time course of vasodilatory responses in skeletal muscle arterioles: Role in hyperemia at onset of exercise. Am J Physiol - Hear Circ Physiol 279:H1715-23. https://doi.org/10.1152/ajpheart.2000.279.4.h1715

122. Hogarth A, Mackintosh A, Mary D (2007) Gender-related differences in the sympathetic vasoconstrictor drive of normal subjects. Clin Sci 112:353–361. https://doi.org/10.1042/CS20060288

123. Hart E, Charkoudian N, Wallin B, et al (2009) Sex differences in sympathetic neural-hemodynamic balance: implications for human blood

pressure regulation. Hypertension 53:571–576. https://doi.org/10.1161/HYPERTENSIONAHA.108.126391

124. Tan CO, Tzeng YC, Taylor JA (2011) Sex differences in sympathetic neurovascular transduction in humans. Auton Neurosci 163:72. https://doi.org/10.1016/J.AUTNEU.2011.05.098

125. Cavalcante JL, Lima JAC, Redheuil A, Al-Mallah MH (2011) Aortic stiffness: Current understanding and future directions. J. Am. Coll. Cardiol. 57:1511–1522

126. Jatoi NA, Mahmud A, Bennett K, Feely J (2009) Assessment of arterial stiffness in hypertension: Comparison of oscillometric (Arteriograph), piezoelectronic (Complior) and tonometric (SphygmoCor) techniques. J Hypertens 27:2186–2191. https://doi.org/10.1097/HJH.0b013e32833057e8

127. Mattace-Raso FUS, Hofman A, Verwoert GC, et al (2010) Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: 'Establishing normal and reference values.' Eur Heart J 31:2338–2350. https://doi.org/10.1093/eurheartj/ehq165

128. Ranjith R, Binu TG, George V, et al (2014) Aortic pulse wave velocity and its relationship with complexity of coronary artery disease based on SYNTAX score. Heart Asia 6:109–115. https://doi.org/10.1136/heartasia-2013-010492

129. Parati G, De Buyzere M (2010) Evaluating aortic stiffness through an arm cuff oscillometric device: Is validation against invasive measurements enough? J. Hypertens. 28:2003–2006

130. Pilt K, Meigas K, Viigimaa M, Temitski K (2011) Possibility to use finapres signal for the estimation of aortic pulse wave velocity. IFMBE Proc 37:524–527. https://doi.org/10.1007/978-3-642-23508-5_136

131. Pilt K, Meigas K, Ferenets R, et al (2014) Photoplethysmographic signal waveform index for detection of increased arterial stiffness. Physiol Meas 35:2027–2036. https://doi.org/10.1088/0967-3334/35/10/2027

132. Liu AB, Hsu PC, Chen ZL, Wu HT (2011) Measuring pulse wave velocity using ECG and photoplethysmography. J Med Syst 35:771–777. https://doi.org/10.1007/S10916-010-9469-0

133. Ouyang V, Ma B, Pignatelli N, et al (2021) The use of multi-site photoplethysmography (PPG) as a screening tool for coronary arterial disease and atherosclerosis. Physiol Meas 42:. https://doi.org/10.1088/1361-6579/ABAD48

134. Charlton PH, Pilt K, Kyriacou PA (2022) Establishing best practices in photoplethysmography signal acquisition and processing. Physiol Meas 43:050301. https://doi.org/10.1088/1361-6579/AC6CC4

135. Obeid H, Khettab H, Marais L, et al (2017) Evaluation of arterial stiffness by finger-toe pulse wave velocity: optimization of signal processing and clinical validation. J Hypertens 35:1618–1625. https://doi.org/10.1097/HJH.0000000000001371

136. Pucci G, Spronck B, Avolio AP, et al (2020) Age-Specific Acute Changes in Carotid–Femoral Pulse Wave Velocity With Head-up Tilt. Am J Hypertens 33:1112. https://doi.org/10.1093/AJH/HPAA101

137. Baier D, Teren A, Wirkner K, et al (2018) Parameters of pulse wave velocity: determinants and reference values assessed in the population-based study LIFE-Adult. Clin Res Cardiol 107:1050. https://doi.org/10.1007/S00392-018-1278-3

138. Haesler E, Lyon X, Pruvot E, et al (2004) Confounding effects of heart rate on pulse wave velocity in paced patients with a low degree of atherosclerosis. J Hypertens 22:1317–1322. https://doi.org/10.1097/01.HJH.0000125447.28861.18

139. Patil SG, Arakeri S, Khode V (2021) Association of Low BMI with Aortic Stiffness in Young Healthy Individuals. Curr Hypertens Rev 17:245–249. https://doi.org/10.2174/1573402117666210121100936

140. Logan JG, Kang H, Kim S, et al (2020) Association of obesity with arterial

stiffness: The Multi-Ethnic Study of Atherosclerosis (MESA). Vasc Med 25:309–318. https://doi.org/10.1177/1358863X20918940

141. Schwartz JE, Feig PU, Izzo JL (2019) Pulse Wave Velocities Derived From Cuff Ambulatory Pulse Wave Analysis. Hypertens (Dallas, Tex 1979) 74:111–116. https://doi.org/10.1161/HYPERTENSIONAHA.119.12756

142. Greve S V., Blicher MK, Kruger R, et al (2016) Estimated carotid-femoral pulse wave velocity has similar predictive value as measured carotid-femoral pulse wave velocity. J Hypertens 34:1279–1289. https://doi.org/10.1097/HJH.0000000000000935

143. Greve S V., Laurent S, Olsen MH (2017) Estimated Pulse Wave Velocity Calculated from Age and Mean Arterial Blood Pressure. Pulse (Basel, Switzerland) 4:175–179. https://doi.org/10.1159/000453073

144. Baulmann J, Schillings U, Rickert S, et al (2008) A new oscillometric method for assessment of arterial stiffness: Comparison with tonometric and piezo-electronic methods. J Hypertens 26:523–528. https://doi.org/10.1097/HJH.0b013e3282f314f7

145. Segers P, Kips J, Trachet B, et al (2009) Limitations and pitfalls of non-invasive measurement of arterial pressure wave reflections and pulse wave velocity. Artery Res 3:79–88. https://doi.org/10.1016/j.artres.2009.02.006

146. Segers P, Mynard J, Taelman L, et al (2012) Wave reflection: Myth or reality? Artery Res 6:7–11. https://doi.org/10.1016/J.ARTRES.2012.01.005

147. Westerhof BE, van Gemert MJC, van den Wijngaard JP (2020) Pressure and Flow Relations in the Systemic Arterial Tree Throughout Development From Newborn to Adult. Front Pediatr 8:251. https://doi.org/10.3389/FPED.2020.00251

148. Ring M, Eriksson MJ olant., Zierath JR a., Caidahl K (2014) Arterial stiffness estimation in healthy subjects: a validation of oscillometric (Arteriograph) and tonometric (SphygmoCor) techniques. Hypertens Res 37:999–1007. https://doi.org/10.1038/hr.2014.115

149. Milan A, Zocaro G, Leone D, et al (2019) Current assessment of pulse wave velocity: Comprehensive review of validation studies. J. Hypertens. 37:1547–1557

150. Rajzer MW, Wojciechowska W, Klocek M, et al (2008) Comparison of aortic pulse wave velocity measured by three techniques: Complior, SphygmoCor and Arteriograph. J Hypertens 26:2001–2007. https://doi.org/10.1097/HJH.0b013e32830a4a25

151. Nemes A, Takács R, Gavallér H, et al (2011) Correlations between Arteriograph-derived pulse wave velocity and aortic elastic properties by echocardiography. Clin Physiol Funct Imaging 31:61–65. https://doi.org/10.1111/j.1475-097X.2010.00980.x

152. Horváth IG, Németh Á, Lenkey Z, et al (2010) Invasive validation of a new oscillometric device (Arteriograph) for measuring augmentation index, central blood pressure and aortic pulse wave velocity. J Hypertens 28:2068–2075. https://doi.org/10.1097/HJH.0b013e32833c8a1a

153. Trachet B, Reymond P, Kips J, et al (2010) Numerical validation of a new method to assess aortic pulse wave velocity from a single recording of a brachial artery waveform with an occluding cuff. Ann Biomed Eng 38:876–888. https://doi.org/10.1007/s10439-010-9945-1

154. Sugawara J, Hayashi K, Yokoi T, Tanaka H (2008) Age-associated elongation of the ascending aorta in adults. JACC Cardiovasc Imaging 1:739–748. https://doi.org/10.1016/J.JCMG.2008.06.010

155. Hart EC, Charkoudian N, Miller VM (2011) Sex, hormones and neuroeffector mechanisms. Acta Physiol 203:155–165. https://doi.org/10.1111/J.1748-1716.2010.02192.X

156. Kneale B, Chowienczyk P, Brett S, et al (2000) Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. J Am Coll Cardiol 36:1233–1238. https://doi.org/10.1016/S0735-1097(00)00849-4

157. Joyner M, Wallin B, Charkoudian N (2016) Sex differences and blood pressure regulation in humans. Exp Physiol 101:349–355. https://doi.org/10.1113/EP085146

158. Jensen-Urstad K, Rosfors S (1997) A methodological study of arterial wall function using ultrasound technique. Clin Physiol 17:557–567. https://doi.org/10.1046/J.1365-2281.1997.00063.X

159. Joyner M, Charkoudian N, Wallin B (2008) A sympathetic view of the sympathetic nervous system and human blood pressure regulation. Exp Physiol 93:715–724. https://doi.org/10.1113/EXPPHYSIOL.2007.039545

160. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ (2005) Flow-mediated vasodilation: A diagnostic instrument, or an experimental tool? Chest 127:2254–2263. https://doi.org/10.1378/chest.127.6.2254

161. Pankevich D, Wizemann T, Altevogt B (2011) Sex Differences and Implications for Translational Neuroscience Research. National Academies Press, Washington, D.C.

162. Harris RA, Nishiyama SK, Wray DW, Richardson RS (2010) Ultrasound assessment of flow-mediated dilation. Hypertension 55:1075–1085

163. Thijssen DHJ, Bruno RM, Van Mil ACCM, et al (2019) Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. Eur Heart J 40:2534–2547. https://doi.org/10.1093/EURHEARTJ/EHZ350

164. Miller A, Cui J, Luck J, et al (2019) Age and sex differences in sympathetic and hemodynamic responses to hypoxia and cold pressor test. Physiol Rep 7:. https://doi.org/10.14814/PHY2.13988

165. McLay KM, Nederveen JP, Koval JJ, et al (2018) Allometric scaling of flow-mediated dilation: is it always helpful? Clin Physiol Funct Imaging 38:663–669. https://doi.org/10.1111/CPF.12465

166. Brod J (1963) Haemodynamic basis of acute pressor reactions and hypertension. Br Heart J 25:227. https://doi.org/10.1136/HRT.25.2.227

167. Gundersen KM, Nyborg C, Heiberg Sundby Ø, Hisdal J (2019) The effects of sympathetic activity induced by ice water on blood flow and brachial artery flow-mediated dilatation response in healthy volunteers. PLoS One 14:e0219814. https://doi.org/10.1371/journal.pone.0219814

168. Zhang X, Zhao S, Li X, et al (2000) Endothelium-dependent and -independent functions are impaired in patients with coronary heart disease. Atherosclerosis 149:19–24. https://doi.org/10.1016/S0021-9150(99)00288-9

169. Gokce N, Keaney JF, Hunter LM, et al (2003) Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. J Am Coll Cardiol 41:1769–1775. https://doi.org/10.1016/S0735-1097(03)00333-4

170. Harms MPM, Wesseling KH, Pott F, et al (1999) Continuous stroke volume monitoring by modelling flow from non-invasive measurement of arterial pressure in humans under orthostatic stress. Clin Sci 97:291–301. https://doi.org/10.1042/CS19990061

171. Bogert LWJ, Van Lieshout JJ (2005) Non-invasive pulsatile arterial pressure and stroke volume changes from the human finger. Exp Physiol 90:437–446. https://doi.org/10.1113/EXPPHYSIOL.2005.030262

172. Christou D, Jones P, Jordan J, et al (2005) Women have lower tonic autonomic support of arterial blood pressure and less effective baroreflex buffering than men. Circulation 111:494–498. https://doi.org/10.1161/01.CIR.0000153864.24034.A6

173. Gordan R, Gwathmey JK, Xie L-H (2015) Autonomic and endocrine control of cardiovascular function. World J Cardiol 7:204. https://doi.org/10.4330/wjc.v7.i4.204

174. Beltrame T, Villar R, Hughson R (2017) Sex differences in the oxygen delivery, extraction, and uptake during moderate-walking exercise transition. Appl Physiol Nutr Metab 42:994–1000.

https://doi.org/10.1139/APNM-2017-0097

175. Hopkins ND, Dengel DR, Stratton G, et al (2015) Age and sex relationship with flow-mediated dilation in healthy children and adolescents. J Appl Physiol 119:926. https://doi.org/10.1152/JAPPLPHYSIOL.01113.2014

176. Levenson J, Pessana F, Gariepy J, et al (2001) Gender differences in wall shear-mediated brachial artery vasoconstriction and vasodilation. J Am Coll Cardiol 38:1668–1674. https://doi.org/10.1016/S0735-1097(01)01604-7

177. Stuckless T, Pyke K (2015) The impact of a cold pressor test on brachial artery handgrip exercise-induced flow-mediated dilation. Vasc Med 20:409–416. https://doi.org/10.1177/1358863X15586473