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The cerebral circulation and oxygenation in response to sympathetic stress in ageing and in patients with metabolic disorders

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DM Diabetes Mellitus

DM1 Type 1 Diabetes Mellitus

DM2 Type 2 Diabetes Mellitus

ESRD End-stage renal disease

MS Metabolic Syndrome

SNS Sympathetic Nervous System

CBF Cerebral Blood Flow

CO Cardiac Output

PaCO2 Partial pressure of arterial carbon dioxide

PaO2 Partial pressure of arterial oxygen

CA Cerebral Autoregulation

SNA Sympathetic Nerve Activity

COX Cerebral Oxygenation

HR Heart Rate

BP Blood pressure

MT Mental Task

EPR Exercise Pressor Reflex

SVR Systemic Vascular Resistance

MAP Mean Blood Pressure

SV Stroke Volume

NST Nucleus of the solitary tract

PEMI Post Exercise Muscle Ischemia

OMS Obesity complicated by metabolic syndrome

MHO Obesity without metabolic syndrome

CTL Healthy Control

SD Standard Deviation

VO2max Maximal oxygen uptake

Wmax Maximum workload

HRmax Maximum heart rate

CER Control exercise recovery

PEP Pre ejection period

VET Left ejection time

DT Diastolic time

VFR Ventricular filling rate

VER Mean systolic ejection rate

SBP Systolic blood pressure

DBP Diastolic blood pressure

NIRS Near infrared spettroscopy

YG Young group

AG Age Group

MCAv Middle cerebral artery blood flow velocity

STAND Standing

WALK15 Walking at 15% of the HRmax

WALK30 Walking at 30% of the HRmax

SIT Seated at rest

TCD Trans-cranial Doppler

CBFv Cerebral blood flow velocity

BMI Body mass index

CVD Cardiovascular disease

CPT Cold pressure test

ROI Region of Interest

CAR% Carotid Artery Response

ΔMAP Maximum increase in MAP

relative ΔMAP Maximum percent increase in MAP

IMT intima-media wall thickness

CHAPTER 1

1 INTRODUCTION

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycaemia caused by a complex interaction of genetics and environmental factors. Depending on the aetiology of the DM, factors contributing to hyperglycaemia include reduced insulin secretion, decreased glucose utilization, and increased glucose production.

The two broad categories of DM are designated type 1 and type 2. Both types of diabetes are preceded by a phase of abnormal glucose homeostasis as the pathogenic processes progress. Type 1 DM (DM1) is the result of complete or near-total insulin deficiency. Type 2 DM (DM2) is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion with a relative insulin deficiency (rather than absolute), and increased glucose production due to a progressive loss of adequate beta-cell insulin secretion frequently on the background of insulin resistance. DM2 is associated with insulin secretory defects related to inflammation and metabolic stress among other contributors, including genetic factors, sedentariety, and ageing. Once hyperglycemia occurs, patients with all forms of diabetes are at risk for developing the same chronic complications, although rates of progression may differ. In detail, the metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems increasing the risk of developing macrovascular and microvascular complications such as end-stage renal disease (ESRD), non-traumatic lower extremity amputations, and adult blindness. Moreover, this disease is associated with an elevate incidence of cardiovascular events, as cardiovascular disease and cerebrovascular disease are the leading cause of morbidity and mortality for individuals with diabetes (American Diabetes Association. Economic costs of diabetes in the U.S. in 2017. Diabetes Care 2018;41:917–928).

My research project focused on DM2, which accounts for 90-95% of all cases of Diabetes (American Diabetes Association. Economic costs of diabetes in the U.S. in 2017. Diabetes Care 2018;41:917–928). Although the specific etiologies are not known, there are several risk factors that cause DM2, such as other metabolic disorders (i.e. obesity and dysplipidemia) and cardiovascular-related conditions often coexisting with DM2. Basically, the risk of developing DM2 increases with age, obesity, and lack of physical activity. For instance, metabolic syndrome (MS) is a metabolic disorder associated with elevated incidences of circulatory abnormalities and cardiovascular events and is often the harbinger of DM2. The International Diabetes Federation (2019) estimated that 20–25% of the global adult population suffers from MS. MS is the term used for individuals whose

glucose levels do not meet the criteria for diabetes but are too high to be considered normal (Selvin et al. 2013). Specifically, subjects who met three or more of the following five criteria were considered as being affected by MS: (1) waist circumference >88 and >102 cm for women and men, respectively; (2) high blood pressure (130/85mmHg); (3) high triglycerides (150 mg/dl); (4) low HDL cholesterol (40 and 50 mg/dl in men and women, respectively); (5) and high fasting glucose level (100 mg/dl) (Alberti et al 2009).

One concerning problem is that DM2 frequently goes undiagnosed for many years because hyperglycaemia develops gradually and, at earlier stages, is often not severe enough for the patient to notice the classic diabetes symptoms. At least initially and often throughout their lifetime, these individuals may not need insulin treatment to survive. Nevertheless, even undiagnosed patients are at increased risk of developing macrovascular and microvascular complications.

A physically active lifestyle has been demonstrated useful in reducing cardiovascular risks. However, the cardiovascular response during exercise can be altered by this disease (Crisafulli et al. 2020, Roberto and Crisafulli 2017). One particular aspect of cardiovascular abnormalities related to DM2 is that this condition has been reported to increase sympathetic nervous system (SNS) tone at rest and during exercise, with elevated blood response to effort (Carnethon et al. 2008, Manzella et al. 2005). Therefore, on one side exercise is considered a valid therapeutic tool for DM2, whereas on the other side these patients may experience troubles in regulating the circulation during effort.

1.1.Cerebral Blood Flow and Cerebral Oxygenation

Normal brain function and survival are the result of an efficient and adequate cerebral blood flow (CBF). The human brain, despite being only 2–3% of the total body mass while requiring 15% of the total cardiac output (CO), consumes 20% of the total oxygen consumption at rest. Given the brain's high energy consumption and lack of meaningful intracellular energy stores, precise control of nutrient supply and by products is essential for the maintenance of CBF. Several mechanisms contribute to regulate CBF, such as cerebrovascular reactivity and cerebral autoregulation, maintaining a constant microvascular perfusion and protecting against deleterious effects of ischemic brain injury and damage (Willie et al. 2011).

According to Poiseuille's law (McDonald 1974), CBF is determined by the cerebral perfusion pressure and the cerebrovascular conductance or its reciprocal, cerebrovascular resistance (Aaslid 1992). Cerebral perfusion pressure is the difference between BP at the level of the circle of Willis

and intracranial pressure; intracranial pressure, in turn, encompasses central venous pressure and the pressures within the cerebrospinal fluid. The variable resistance to flow is encountered mostly in the cerebral arteriolar and capillary bed (Edvinsson et al. 2002). In contrast, the large cerebral arteries and veins are dedicated to blood distribution and storage of blood volume and are, in principle, noncompliant and act merely as a conduit for the pulsatile arterial flow from the aorta to the brain (Olufsen et al. 2002).

Arterial blood gases, BP, cerebral metabolism and neurogenic innervation influence CBF at rest (Busija et al. 1984). It is well established that the cerebral vasculature is strongly affected by partial pressure of arterial carbon dioxide ($PaCO_2$) (Brian et al. 1996). The interaction between $PaCO_2$ and vasodilation/vasoconstriction is normally ascribed to occur at the level of the arterioles and the precapillary sphincters (Atkinson et al. 1990, Edvinsson et al. 2002). Increased CO_2 results in a relaxation of the vascular smooth muscle of all cerebral vessels, although the small vessels are the most responsive (Wei et al. 1980). By contrast, the vasoconstrictor effect of hypocapnia is unaffected by vessel size (Wei EP et al 1980). On the other hand, the role of partial pressure of arterial oxygen (PaO_2) in the day-to-day regulation of CBF seems to be minor, reflected in the findings that, depending on the prevailing $PaCO_2$ (Ainslie et al. 2004), a drop in PaO_2 below a certain threshold (40 mmHg) is required for cerebral vasodilation (Gupta et al. 1997).

Cerebral auto-regulation (CA) adjusts cerebral arteriolar caliper, or cerebrovascular resistance, to ensure that CBF levels are matched to metabolic needs, and it comprises two main components: static and dynamic. Static CA keeps CBF constant over gradual and progressive changes in cerebral perfusion (Paulson et al. 1990). Dynamic CA refers to the rapid regulation of CBF in response to changes in BP that occur in a few seconds (Zhang et al. 2002). Although the cerebral circulation is richly innervated with sympathetic nerve fibres, the effect of sympathetic nerve activity (SNA) on the regulation of CBF remains controversial (Van Lieshout et al. 2008). Traditionally the increase in sympathetic activity appears to have a limited effect on the cerebral vasculature of humans, particularly at rest (Harper et al. 1972). It seems likely that any potential influence of SNA on CBF regulation is masked by the other more powerful regulatory influences on CBF, i.e. auto-regulation (Levine BD et al 2008). However, recently it has been reported that sympathetic activity influences the reactivity of CBF to $PaCO_2$ (Jordan et al. 2000, D'Alecy et al. 1979). Thus, although the influence might be regarded as minor, CO_2-induced elevations in SNA have the potential to affect CBF by direct and indirect mechanisms.

1.2 Cerebral Blood Flow and Cerebral Oxygenation in Aging

It is well accepted that ageing is associated with reductions in regional and global resting cerebral CBF (Scheel et al. 2000, Beason-Held et al. 2007, Ainslie et al. 2008). Resting cerebral energy metabolism is also reportedly altered, such that cerebral oxygenation (COX) and glucose consumption are both reduced (Leenders et al. 1990), although this has not been a universal finding (de Leon et al. 1984). In recent studies an increased risk for development of cognitive impairment that can occur later in life was also associated with a lower cerebral blood flow or perfusion (Yew and Nation 2017, Hampstead et al. 2018), as well as a higher risk of brain atrophy in elderly individuals (Loehrer et al. 2015). A reason for this might be the loss of endothelial function and the age-related stiffening of artery walls (Beaudin et al. 2014, Tarumi and Zhang 2018).

Age-related deficits in the brain are likely to become more evident during physiological perturbation, but the influence of ageing on CBF, COX and metabolism during exercise remains incompletely understood, although perturbations of COX and metabolism have been reported to prevent full activation of exercising skeletal muscles (i.e. provoke central fatigue) (Rasmussen et al. 2010). However, recent findings indicate that physical activity (i.e. low to moderate exercise) has a direct impact on CBF and COX (Smith and Ainslie 2017) and may protect against the age related alterations in cerebral function and cognitive decline (Coetsee and Terblanche 2017).

1.3 Cerebral Blood Flow and Cerebral Oxygenation in response to sympathetic stress

The primary functions of the human circulation during whole-body aerobic exercise are as follows: (1) to ensure adequate delivery of oxygen and metabolic nutrients to fulfil the increased demand of tissue metabolism and the consequent removal of metabolic end-products; and (2) to regulate systemic BP such that adequate organ-specific perfusion pressure is met. During exercise, however, CBF, COX, BP, heart rate (HR), CO and metabolism all increase, albeit each with different magnitudes. However, it is well accepted that the primary factor involved in regulating CBF during exercise is $PaCO_2$ which increases and decreases based on the level of alveolar ventilation determined by exercise intensity. In addition, the balance between sympathetic and parasympathetic neural control of the entire cardiovascular system is changed during exercise (White & Raven, 2014).

Exercise of low (25% Wmax) to moderate intensity (50% Wmax) is characterized by an increase in CBF until 60 % of maximal oxygen uptake (Schroeder et al. 1989), whereas at high intensity CBF

tends to plateau or to progressively decrease, depending on the level of $PaCO_2$ (Smith and Ainslie 2017). Exercise can influence cognitive functions by increasing brain activation and cerebral blood flow and perfusion (Dupuy et al. 2015). Exercise increases (on average of one-half standard deviation) cognitive performance, independently of the cognitive task, the characteristics of participants, and the training method (Colcombe and Kramer, 2003). Executive function enhancement was reported after 6 months of aerobic training (Baker et al. 2010), while functional plasticity of response inhibition process improved after 12 months of resistance training (Liu-Ambrose et al. 2012). Mental activity is also characterized by an increase in CBF and COX, as a result of the neural activation to meet the increased demand for oxygen and substrates (Coetsee and Terblanche 2017). It has been demonstrated that a dual task (i.e. physical activity/cognitive stimulus) causes major demands for cognitive resources (Gatouillat 2015), which may also lead with an increase in CBF and COX. However, the overview of the impact of low-intensity physical activity and mental activity on cerebral perfusion is still lacking.

1.4 Cerebral Oxygenation and Metabolic Disease

Decrements in COX have been related to the development of central fatigue (González-Alonso et al. 2004, Rasmussen et al. 2010, Secher et al. 2008). It has been observed that, during exercise, CBF is impaired in patients with DM2 and that this is related to the elevated SNA. This phenomenon may reduce COX and central motor drive, thereby inducing central fatigue (Kim et al. 2015, Vianna et al. 2015). It was recently observed that subjects suffering from MS cannot properly enhance COX when a mental task (MT) superimposes the metaboreflex, i.e. a cardiovascular reflex activated whenever we exercise (Doneddu et al. 2020). Taking into consideration that MS and is often the harbinger of DM2 (Eckel et al. 2005), it can be hypothesized that, similarly to that previously observed for MS, COX impairment is also present in DM2 patients. This may be of clinical importance also taking into consideration that patients with DM2 may experience SNS dysfunction even worse compared to those with MS (Istenes et al. 2014). Furthermore, this phenomenon may have clinical implication. Indeed, during exercise metaboreflex and MT are contemporaneously present. If in DM2 patients COX does not properly increase, then it is possible that they experience disproportionate fatigue. This phenomenon may provide the basis for the poor predisposition to exercise often observed in individuals with metabolic disorders such as DM2. Given the high incidence of cardiovascular complications of DM2 and the related social burden, it would be of interest to ascertain whether in these patients the COX response during contemporary metaboreflex

and MT resembles what reported in MS. To the best of my knowledge, none has to date investigated this topic.

1.5 Regulation of Cardiovascular response to Exercise in Healthiness

During dynamic exercise the cardiovascular system is adjusted on the basis of certain neural mechanisms. In detail, one is a feed-forward mechanism arising from regions of the brain responsible for motor unit recruitment. It is commonly known as "central command" and it establishes a basal level of sympathetic tone and parasympathetic withdrawal closely linked to the intensity of effort. This basic autonomic activity is in turn finely adjusted by the "exercise pressor reflex" (EPR). This latter reflex works on the basis of peripheral signals arising from type III and IV nerve endings in the muscle, which convey to the cardiovascular control centres information about the mechanical and metabolic conditions of the contracting muscle (Crisafulli et al. 2015, Nobrega et al. 2014, Rowell and O'Leary 1990). The metabolic part of the EPR is commonly termed the muscle "metaboreflex". The metaboreflex has been demonstrated to be dysregulated in several cardiovascular and metabolic diseases, including DM2 (Crisafulli 2017, Roberto et al. 2019, Vianna and Fisher 2019).

The mechanism involved in controlling the cardiovascular apparatus have to deal with two main tasks: (1) to provide adequate oxygen to fulfil metabolic demand of exercising muscles and to guarantee metabolic end-products washout; and (2) to regulate arterial blood pressure in order to maintain adequate perfusion of the vital organs, such as the brain, without excessive pressure variations (Ichnose et al. 2014). Physical activities that involves large muscle mass such as running, cycling, and rowing produce an intense vasodilation in the working muscle due to the accumulation of metabolic by-products. Local metabolites production overwhelms the exercise-induced increase in sympathetic tone, leading to a reduction in systemic vascular resistance (SVR), a phenomenon called *functional sympatholysis* (Murphy et al 2011).

Metabolite induced vasodilation (*i.e.* SVR reduction) is a challenge for cardiovascular regulation because it would cause a drop in blood pressure. However, in healthy subjects, cardiovascular control mechanisms increase CO, thereby keeping mean blood pressure (MAP) at a stable level or even moderately increasing it, so that cerebral perfusion is normally preserved (Lewis et al. 1983). Dynamic exercise is characterized by a small increase in MAP, whereas during static/isometric exercise there is a well-established progressive increase in MAP. This small MAP increment induced by dynamic exercise takes place despite the profound SVR reduction due to the metabolic-induced vasodilatation (Crisafulli et al. 2006). The increase in CO is achieved thanks to an increase

in HR and stroke volume (SV), which counteract the reduction in SVR via a flow-increment mechanism, that is, by a rise in CO (Figure IA).

In healthy individuals, the described cardiovascular regulation is highly effective and the target blood pressure can often be achieved despite a lack in response of one of these regulated variables (Crisafulli et al. 2002). Concerning mechanical mechanisms, both respiratory and skeletal muscle pumps contribute to the increase in SV and CO occurring during dynamic exercise. As regards the neural component of this regulation, exercise induces parasympathetic withdrawal and sympathetic activation, which are a function of exercise intensity and the muscle mass recruited. Parasympathetic withdrawal aims at increasing HR, while the engagement of the sympathetic activity aims at increasing HR, at enhancing myocardial contractility to increase SV, at inducing venoconstriction to improve venous return, at increasing vascular resistance in the abdominal viscera and non-active skeletal muscles, and at preserving most of the available CO for the perfusion of the active muscle, where metabolic-mediated vasodilation takes place. There are at least three neural mechanisms contributing to this cardiovascular adjustment: 1) *central command*, 2) *exercise pressor reflex*, and 3) *arterial baroreflex* (Nobrega et al. 2014, Crisafulli et al. 2015). One is a central mechanism, while the others two origin in the contracting muscle.

1.5.1 Central Command

The central command consists of the activation of regions of the brain responsible for somatomotor activation that also impinge on the cardiovascular control areas located in the medulla, mediating autonomic responses that are crucial for cardiovascular regulation during exercise. The *medulla oblongata* contains the major nuclei that control blood pressure and the cardiovascular system and is responsible for the integration and elaboration of signals arising from these three neural mechanisms (Green et al. 2008). It is believed that the "central command" sets a basal level of sympathetic activity and vagal withdrawal closely related to the intensity of the strain and to motor drive from the motor cortex (O'Leary 1993). This autonomic modulation is then adjusted by the exercise pressor reflex on the basis of peripheral signals arising from mechano- and metabo-receptors (type III and IV nerve endings) within the muscle. In detail, it is known that groups III and IV nerve endings excite neurons in the nucleus of the solitary tract (NST) in the medulla. Sympathetic stimulation is in turn modulated by baroreflexes, which oppose any mismatch between vascular resistance and CO by controlling muscle vasodilatation and cardiac chronotropism in order to avoid excessive variation in BP (Fadel et al. 2001).

Summing up, the central command is traditionally considered a feed-forward mechanism, which consists of neural impulses from the motor cortex that irradiate to autonomic neurons in the brain stem, leading to parasympathetic withdrawal and sympathetic activation.

1.5.2 Exercise pressor reflex

Alam and Smirk (1937, 1938) provided the first evidence that metabolic reflex arising from skeletal muscle adjusts hemodynamics during exercise. Following this seminal research, a great bulk of evidence demonstrated that group III/IV afferents within the muscle gather information about the metabolic condition of the working muscle and are responsible for cardiovascular reflex (Nobrega et al. 2014, Kaufman et al. 1983). This reflex was then termed *muscle metaboreflex*. It was later also demonstrated that mechanical changes in muscles and tendons elicit cardiovascular reflex, and this was termed *mechanoreflex*. These two reflexes of muscular origin together constitute the *exercise pressor reflex*. It is well established that these two reflexes have their afferent arm in groups III and IV nerve endings within the muscle, with type III nerve afferents mainly acting as mechanoreceptors and type IV as metaboreceptors (Andreani et al. 1997). It is however important to underline that this classification is not imperative and that both fiber types can act dually as metabo- and mechanoreceptors.

Type III/IV afferents act as receptors and collect information concerning the mechanical and metabolic conditions of contracting muscles. This information is then sent to cardiovascular controlling centres located in the *medulla oblongata*, where the information is integrated and elaborated. In turn, the cardiovascular medullary centres organise the hemodynamic response to exercise on the basis of the mechanical and metabolic status of the working muscle. From a hemodynamic point of view, the typical consequence of metaboreflex recruitment is an increase in sympathetic tone and MAP (Piepoli et al. 1995, Crisafulli et al. 2003).

In healthiness, in order to reach the target MAP, the metaboreflex recruits the functional reserve of all four hemodynamic modulators: myocardial contractility, cardiac preload, afterload and chronotropism (Crisafulli et al. 2003, Nobrega et al. 2014). Thus, from the available literature it can be gleaned that the hemodynamic response to metaboreflex activation is a highly integrated phenomenon where a complex interplay between cardiac performance, preload, afterload, and HR occurs to achieve the normal cardiovascular response to exercise. It should be noticed that research

on the mechanoreflex is less abundant than that on metaboreflex and a clear and complete picture of the hemodynamic consequences of pure mechanoreflex activation is still lacking.

In summary, from available data it seems that the exercise pressor reflex can adjust all four hemodynamic modulators (i.e., chronotropism, inotropism, cardiac preload, and afterload) to reach the target BP during exercise. However, while the metaboreflex contribution to this reflex is well characterized, less is known about the hemodynamic effects of mechanoreflex activation.

1.5.3 Arterial Baroreceptors

Arterial baroreceptors are located at the medial-adventitial border of blood vessels in the carotid sinus bifurcation and aortic arch. They are pivotal in inducing the rapid adjustments that occur during acute cardiovascular stress via control over HR and peripheral vascular responses to changes in arterial pressure (Sagawa and Eisner 1975, Fadel and Raven 2012). When arterial BP is acutely elevated or reduced, the baroreceptors are stretched or compressed and this deformation leads to an increase or a decrease in afferent neuronal firing, respectively. These afferent neural responses via baroreceptors result in reflex-mediated systemic neural adjustments with changes in sympathetic and parasympathetic nerve activities. This autonomic modulation affects both central (cardiac) and peripheral (vessels) circulation in order to return BP to its original operating pressure point.

Recent findings suggest that the carotid baroreflex is reset during dynamic exercise and it functionally operates around the exercise-induced increase in BP (Iellamo et al. 2001, Ogoh et al. 2005). The “resetting” of the arterial baroreflex is essential to evoke and maintain an effective autonomic nervous system modulation and an adequate cardiovascular adjustment to exercise. The baroreflex acts to finely balance the opposing effects of sympathetic vasoconstriction and metabolic vasodilation, and it also acts to partly restrain the BP response to exercise by buffering activation of the increase in sympathetic activity due to the central command and the exercise pressor reflex.

1.5.4 Reflexes interaction during exercise

During exercise, exercise pressor reflex, central command, and baroreflex are all activated and complex interaction occurs between these reflexes. While it is well ascertained that some redundancy and neural occlusion exist between exercise pressor reflex and central command (i.e., their effects do not sum), it is also remarkable that they can all modulate the activity of the other two. Moreover, it is now well established that both central command and the exercise pressor reflex are involved in the mechanism of baroreflex resetting during exercise (Figure IB).

Central command, as a feed-forward mechanism, is likely to be the primary regulator of exercise-induced baroreflex resetting, whereas the exercise pressor reflex operates mainly as a feed-back mechanism. Thus, it exerts a more modulatory role. Furthermore, it seems that both inputs interact and are important for the complete exercise-induced baroreflex resetting (Fadel and Raven 2012). The interaction between reflexes clearly appears during post exercise muscle ischemia (PEMI), a method usually employed to study the cardiovascular effects of metaboreflex activation (Crisafulli et al. 2003, Amann et al. 2011). During PEMI, there is normally no HR response notwithstanding the activation of exercise pressor reflex and the augmented sympathetic activity. The absence of HR response in this setting is the consequence of the fact that the rise of sympathetic activity due to metaboreflex activation is counteracted by the concomitantly augmented parasympathetic outflow due to the central command deactivation and the concomitant enhanced arterial baroreflex activity that buffers the metaboreflex-mediated increase in MAP (Crisafulli et al. 2011, Nishiyasu et al. 1994).

Thus, if the metaboreflex is activated by the PEMI method, the elevated sympathetic activity to sinus node is counteracted by enhanced parasympathetic tone due to the withdrawal of central command and to the sympathetic-buffering effect of baroreflex activation. This fact is not evident when metaboreflex is activated during exercise when central command is operating (Crisafulli et al. 2001), thereby indicating that central command acts as a modulator of baroreflex activity during exercise.

To summarise, during exercise the hemodynamic status is controlled by the nervous system by integrating signals originating from the brain (central command) and from the periphery (exercise pressor reflex, and baroreflexes). In healthy individuals, the final result is that sympathetic activity to cardiovascular apparatus augments and prevails over parasympathetic tone, which conversely declines. As a consequence, HR and myocardial contractility increase, while there is arterial constriction in the vascular beds of organs and tissues not involved in exercise. Furthermore, there is an increase in cardiac preload, due to muscle pump activation and sympathetic-induced venoconstriction, which together contribute to the SV increment commonly observed during dynamic exercise.

1.6 Hemodynamics Dysregulation in Metabolic Disease

Situations such as cardiovascular dysfunctions (hypertension) and metabolic diseases (DM1, DM2 and MS) have all been associated with metaboreflex malfunction and abnormal hemodynamic response (Murphy et al. 2011, Roberto et al. 2012, Roberto et al. 2019, Milia et al. 2015, Delaney et al. 2010, Milia et al. 2015). These clinical conditions are characterized by excessive sympathetic tone and pronounced arteriolar constriction that causes exaggerated SVR increments, thereby leading to ventricular afterload elevation in response to the metaboreflex (Delaney et al. 2010, Murphy et al. 2011). This phenomenon limits ventricular pumping and reduces SV during the metaboreflex. Therefore, in these cases, the exaggerated vasoconstriction is not the consequence of deficits in inotropism and/or preload. Rather, it is because of an elevated sympathetic tone *per se*. Several studies suggest that, similarly to what was observed for MS, in DM2 there is an exaggerated sympathetic tone during exercise and during the metaboreflex, with elevated vasoconstriction and blunted vasodilator response (Roberto et al. 2012).

This kind of response is also observed in a number of other cardiovascular diseases, thus supporting the point of view that DM2 can be considered a cardiovascular disease (Schillacci et al. 2006). Recently, Roberto et al. (2019) demonstrated that patients with DM2 exaggeratedly increased SVR (i.e., increased arteriolar vasoconstriction) in response to the metaboreflex activation as compared with healthy controls, whereas SV and CO response were blunted. In these patients, BP response to the metaboreflex relies more on SVR increases rather than on cardiac performance adaptations. These results closely resemble what was observed in patients with MS (Milia et al. 2015, Murphy et al. 2011) and support the concept that in patients suffering from metabolic disorders such as obesity, MS, and DM2, exaggerated arterial vasoconstriction and reduced vasodilator responsiveness are present during exercise and during metaboreflex stimulation (Vianna et al. 2015). A recent study by Milia et al. (2015) showed that during the metaboreflex, subjects suffering from obesity complicated by metabolic syndrome (OMS) exhibited a hemodynamic response mainly characterized by a pronounced increase in arteriolar vasoconstriction with respect to both obesity without metabolic syndrome (MHO) and normal controls (CTL). Moreover, the OMS group had a reduced CO response in comparison with the CTL group, whereas this phenomenon was not present in subjects of the MHO group.

Taken together, these findings suggest that in obesity complicated with metabolic syndrome, the blood pressure adjustments during the metaboreflex are orientated toward vasoconstriction and excessive increase in SVR without any significant CO participation. Thus, this finding suggests that

these patients had the tendency to exaggeratedly augment sympathetic tone and to decrease the parasympathetic tone during the metaboreflex activation. This fact is in accordance with the concept that obesity, especially if complicated with metabolic syndrome, is accompanied with autonomic dysfunction with sympathetic overdrive (Xiong et al. 2014).

1.7 AIM OF THE RESEARCH

Notwithstanding the extensive scientific research on the CBF and COX response to sympathetic stress in healthy individuals at rest, the effect of daily life activities (DLA), such as low-intensity exercise and mental stress, in both healthy individuals and in patients with metabolic diseases (i.e. MS and DM2) are still unclear and require further investigation. Moreover, given the high incidence of cardiovascular complications of DM2 and the related social burden, it would be of interest to ascertain whether in these patients the CBF, the COX, and the cardiovascular response during DLA-induced sympathetic activation resemble what already reported in MS (Doneddu et al. 2020) and aging (Milia et al. 2015).

In particular, the main focus of my research was to elucidate the interaction between CBF/COX and metaboreflex activation in response to sympathetic stress during DLA (i.e. mental activity, low-intensity exercise) in healthy subjects and in patients with DM2.

To reach this goal, I devised and conducted two experiments in which CBF and COX were studied during sympathetic activation in response to PEMI during MT (Experiment 1) and during DLA (Experiment 2)(Figure II).

Moreover, I performed a third study to better understand the link between BP and carotid artery vasomotion. Specifically, I investigated the relationship between the timing and magnitude of the sympathetically-induced elevation in BP and carotid artery diameter responses in healthy, middle-aged men (Experiment 3). This experiment was set to understand the relationship between the increase in blood pressure and carotid artery diameter in response to sympathetic stimulation in healthy, middle-aged men (Figure II).

Regarding Experiment 1, I hypothesised that in DM2 patients COX did not properly increase during sympathetic activation induced by contemporary metaboreflex and mental task, and this may lead to disproportionate fatigue, thereby providing a potential basis for the poor predisposition to exercise often observed in individuals with metabolic disorders such as DM2.

Furthermore, since low-intensity exercise in daily living may alter cerebral perfusion, such effects may be altered by older age. In Experiment 2, I hypothesised that daily life low-intensity physical activities showed immediate changes in cerebral perfusion. Furthermore, I compared these changes between young and older individuals.

Finally, in experiment 3 I investigated on the potential link between blood pressure and carotid artery vasomotion. Specifically, I investigated the relationship between the timing and magnitude of the sympathetically-induced elevation in blood pressure and carotid artery diameter responses.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Experiment 1

2.1.1 Study population.

Two groups of subjects were studied:

- DM2 group, composed by 13 patients [6 females, mean ± standard deviation (SD) of the mean of age 50.5±10.3 years]. They were recruited on the basis of the following criteria: clinical history of DM2 for at least 1 year (range 1-6 years), with stable metabolic condition (HbA1c level <9% at the time of the study), and absence of signs or symptoms of peripheral neuropathy. All patients were on medication with oral hypoglycaemic agents and 11 with insulin. Seven of them were also under medication for high blood pressure (3 with sartans and 4 with ACE-inhibitors) and 8 for high cholesterol (statins).

- Control (CTL) group, composed by 13 age-matched healthy subjects (6 females, mean ± SD of age 49.3±7.5 years), without any known metabolic disease as resulted from anamnesis and physical examination.

For both groups further exclusion criteria were: age ≤18 and ≥ 65 years, presence of any associated medical conditions that could interfere with the autonomic function and/or chronic cardiopulmonary diseases. Smokers and patients taking β-blockers, sympatho-mimetics, and/or tricyclic antidepressants were also excluded from both groups. All women were tested during the follicular phase of their menstrual cycle (i.e. within 10 days from the start of menstruation) as self-reported.

Written informed consent was obtained from all participants. The study was approved by the local ethical committee and conforms to the declaration of Helsinki.

2.1.2 Experimental design.

Preliminary visit. After enrolment, participants underwent a preliminary examination with anamnesis, medical visit, and anthropometric measurement. Then they performed a cardiopulmonary test to assess their maximal oxygen uptake (VO2max), maximum workload (Wmax), and maximum heart rate (HRmax). In detail, the cardiopulmonary consisted in an incremental exercise on an electromagnetically-braked cycle ergometer (CUSTO Med, Ottobrunn, Germany), with a linear increase in workload (10W/min), starting at 10W, at a pedalling frequency

of 60 rpm. The cardiopulmonary was conducted until exhaustion, which was considered as the workload at which the subject was unable to maintain a pedalling rate of at least 50 rpm. To measure VO2max, subjects were connected with a face mask to a metabolic chart (ULTIMA CPX, MedGraphics St. Paul, MN) calibrated immediately before each test. Achievement of VO2max was considered as the attainment of at least 2 of the following criteria: 1) a plateau in VO2 despite increasing workload (<80 mL•min-1); 2) respiratory exchange ratio above 1.10; and 3) HR ± 10 beats·min-1 of predicted HRmax calculated as 220-age. In this occasion, maximum handgrip strength of participants was also assessed as the peak reached during 5 maximal compressions on a hydraulic dynamometer (MAP 1.1, Kern, Balingen, Germany) in the non-dominant harm. This preliminary visit allowed subjects to familiarise with the staff, equipment, and procedures of the experimental session described in the next paragraph.

2.1.3 Experimental session.

After an interval from the preliminary visit of at least 3 days (range 3-7 days), each participant reported to the laboratory and underwent the following protocol assigned in random order while seated on a chair. Randomisation was obtained with an online random sequence generator (https://www.random.org/sequences/).

a) PEMI session. In this session, subjects rested for three minutes seated on a chair. Then, they performed three minutes of rhythmic (30 compressions/min) dynamic handgrip in the non-dominant harm using the same dynamometer employed in the preliminary visit; the workload was 30% of the maximum. Handgrip was followed by three minutes of PEMI on the exercised arm. Muscle ischemia was induced by rapidly (in less than three seconds) inflating an upper arm biceps tourniquet to 50 mmHg above peak exercise systolic pressure. The tourniquet was kept inflated for three minutes. Three minutes of further recovery were allowed after the tourniquet was deflated, for a total of six minute of recovery. The PEMI manoeuvre has been utilised several times in healthy individuals as well as in patients with cardiovascular and metabolic diseases in our previous investigations dealing with the metaboreflex. This manoeuvre has been demonstrated capable of eliciting the metaboreflex-induced hemodynamic stimulation and to detect cardiovascular abnormalities (Crisafulli et al. 2017).

b) control exercise recovery (CER) session. Participants performed the same rest-exercise protocol used for PEMI, but recovery was without tourniquet inflation, i.e. without metaboreflex activation.

c) MT session. During this session, subjects were engaged in a MT at rest. The MT started after a rest period of six minutes and lasted for three minutes. Three minutes of further recovery were allowed after the MT ceased. Thus, the MT was conducted at the same time-point and had the same duration of the muscle ischemia of the PEMI test. In detail, the MT was the Bivalent Shape Task (Esposito et al. 2013), which is a computerised attentional interference test constructed to test the ability to suppress interference. The test was performed using a cross-platform open source programming language designed to implement both simple and complex psychological tests. The MT required the participant to determine whether a shape at the centre of the screen was a circle or a square by moving a computer mouse with the dominant no-exercising hand. Visual response cues were shaded in either red, blue or an unfilled black outline. In all cases, the color was irrelevant and not used to make the decision.

d) CER+MT session. The same rest-exercise protocol employed in CER test was performed, but the exercise phase was followed by a MT session of three minutes. Three further minutes of recovery were allowed after the termination of MT. Thus, this session had the same duration of the MT session described at point c), but exercise was conducted before the MT.

e) PEMI+MT session. The same rest-exercise protocol used for PEMI was performed, but handgrip was followed by three minutes of contemporary PEMI and MT. Three minutes of further recovery were allowed after the termination of the PEMI+MT sessions.

Figure 1 schematises the various sessions of the study protocol. As it can be gleaned, all the sessions were composed of four blocks each lasting three minutes, for a total of 12 minutes. Sessions were spaced by at least 15 minutes of recovery, which was considered complete when HR was not higher than 5 bpm compared to the pre-exercise level. This protocol has been recently used in a similar investigation in patients suffering from MS (Doneddu et al. 2020).

All the experiments were carried out in a temperature-controlled, air-conditioned room (temperature set at 22°C and relative humidity 50%). Participants were requested to abstain from caffeinated beverages, alcohol, and heavy exercise for 12 h prior to reporting to the laboratory.

2.1.4 Assessment of hemodynamics and cerebral oxygenation.

Throughout all the tests, hemodynamic variables were measured using the trans-thoracic impedance method. This method assumes that changes in trans-thoracic impedance is representative of SV, which was estimated with the Sramek-Bernstein equation (Bernstein 1986). This kind of hemodynamic assessment has been previously used in several investigations in similar experimental

settings and the data acquisition and elaboration processes are described in previous research (Crisafulli et al. 2008, Crisafulli et al. 2009, Crisafulli et al. 2013). Briefly, traces of electrocardiogram, thorax impedance (Z0), and Z0 first derivative were acquired with an impedance cardiograph (NCCOM 3, BoMed Inc., Irvine, CA). Traces were stored with a digital chart recorder (ADInstruments, PowerLab 8sp, Castle Hill, Australia) and then SV and HR were calculated from stored traces. CO was obtained by multiplying SV by HR. The pre ejection period (PEP) and the left ejection time (VET) were also assessed from stored traces, as previously described (Sainas et al. 2016).

By subtracting the sum of PEP and VET periods from the cardiac cycle duration, diastolic time (DT) was calculated (Gledhill et al. 1994). To have a measure of the mean rate of diastolic blood flux, SV was divided by DT, thereby obtaining the ventricular filling rate (VFR) (Crisafulli et al. 2009, Gledhill et al. 1994, Milia et al. 2015). Furthermore, we calculated the SV/VET ratio. This parameter is the mean systolic ejection rate (VER), which is directly related to myocardial performance (Gledhill et al. 1994, Sanna et al. 2017).

Systolic (SBP) and diastolic (DBP) blood pressure values were assessed each minute using a standard manual sphygmomanometer placed in the non-exercised arm. Blood pressure was measured by the same physician throughout all the sessions of the study. MAP was calculated by taking into consideration changes in the diastolic and systolic periods (Sainas et al. 2016). SVR was assessed as the MAP/CO ratio multiplied by 80, where 80 is a conversion factor to change units to standard resistance units.

During experiments, COX was estimated by employing the near infrared spectroscopy (NIRS; Nonin, SenSmart X-100, Plymouth, MN, USA). This technique estimates oxygenated Hb in the brain tissue with values that are representative of the frontal cortical microcirculation in the grey matter, including arteries and veins. Two NIRS sensors were placed on the left and right side of the subject's forehead above the eyebrow, in the regions between Fp1 and F3 (international EEG 10-20 system) and adjusted according to the strength of the signal. The probes were covered and held in place using a headband and were taped to reduce the intrusion of extraneous light. Particular care was taken to ensure that probes did not constrict the head, so blocking the local circulation. Since the absolute concentration of oxygenated Hb could not be obtained because the path length of NIRS light in the brain tissue was unknown, relative changes of NIRS signals against the baseline values were taken into account for calculation. As previously reported, oxygenated Hb measured obtained with NIRS may be considered an index of regional tissue blood flow (Asahara et al. 2016, Ishii et

al. 2018, Suzuki et al. 2004). It is accepted that changes in COX are representative of cortical activation (Strangman et al. 2002a, Strangman et al. 2002b). The NIRS method has already been utilised for COX assessment during mental tasks (Doneddu et al. 2020, Plichta et al. 2006, Strangman et al. 2002a, Verner et al. 2013). The accuracy and reliability of the method were validated against a mix of 70% jugular bulb saturation and 30% arterial saturation (MacLeod et al. 2012).

2.1.5 Data analysis and calculation.

Data are presented as means ± SD. The Kolmogorov-Smirnov test was employed to determine whether variables were normally distributed. To calculate the required sample size, we employed a calculation with a power of 85%, an overall type 1 error of 0.05 (two sided), and a 20% difference between groups in the studied variables. Twelve subjects were needed to obtain adequate statistical power.

Differences between groups in anthropometric characteristics and in parameters of the CPT test were found out with the t test for unpaired data. The hemodynamic data gathered during the five sessions of the study protocol were averaged over one minute. The differences between the groups in absolute values at rest and at the third minute of exercise (i.e. the 6th minute of the sessions) were tested using the two-way analysis of variance (ANOVA, factors: group and condition) followed by Bonferroni post-hoc when appropriate. The same statistics were applied to the 9th minute of the sessions, which corresponded to the last minute of PEMI, MT, PEMI+MT, and CER+MT tests, i.e. when a steady state in variables values was supposed to be reached. Two-way ANOVA (factors: group and condition) was also applied to changes in COX, which, as previously explained, were reported as percentage changes from rest.

Statistics were carried out using commercially available software (GraphPad Prism). Significance was established as a p value of <0.05 in all cases.

2.1.6 Results

The protocol was completed by all the subjects and none of the participants reported unbearable discomfort or pain during the PEMI manoeuvres. All the collected variables were normally distributed and were analysed using parametric tests.

Table 1 shows the anthropometric data, results of the screening test and of the CPT. Individuals in the DM2 group had higher body mass, BMI, SBP, and fasting glucose with respect to the CTL group, whereas VO2max, Wmax, and HRmax were lower.

Table 2 reports hemodynamic and COX data collected during the rest periods of the five protocol sessions. While conditions did not affect any of the studied parameters, statistics discovered that on average HR, SVR, and MAP were higher in the DM2 than the CTL group; differently, SV, VER and COX were lower. No significant interaction was found for any variable.

Table 3 shows that during the handgrip test the DM2 group showed on average higher HR, SVR, and MAP and lower SV and VER values in comparison with the CTL group. Moreover, MAP, PEP, and COX were significantly affected by the condition. In detail, in both groups during the MT test (i.e., a condition when there was neither exercise nor mental task at this time point in the protocol) MAP was lower than during the other protocol sessions. PEP tended to shorten during the exercise sessions in comparison with the MT test, reaching statistical significance during the CER+MT and the PEMI+MT tests of the CTL group and during the CER test of the DM2 group. In both groups COX significantly increased during the CER, the PEMI, the CER+MT, and the PEMI+MT tests as compared to the MT test.

Figures 2-4 show the values of the hemodynamics and COX collected at the 9th minute of recovery during the sessions. In this period, a steady state in variables' level was supposed to have been achieved in response to the PEMI and the MT manoeuvres.

Figure 2 (panel A) shows that on average HR was higher in the DM2 in comparison to the CTL group. Moreover, SV and CO (panels B and C respectively) were lower in the DM2 than in the CTL group. There was neither condition nor interaction effect for any of these variables.

VFR (Figure 3, panel A) was not different between groups, whereas VER (panel B) was on average higher in the CTL in comparison to the DM2 group. There was no condition or interaction effect for both VFR and VER. MAP was on average higher in patients with MS compared to CTL subjects. Moreover, there was a condition effect. Post-hoc analysis found out that in the DM2 group, MAP was higher in the PEMI and in the PEMI+MT than in the CER session. No interaction effect was discovered for MAP.

Figure 4 (panel A) illustrates that the level of SVR was higher in the DM2 than in the CTL group, without any condition or interaction effect. Panel B of Figure 4 demonstrates that there was no difference between the two groups in terms of PEP. Statistics discovered a significant condition effect. The post-hoc analysis found out that in both groups PEP was lower in the PEMI and in the PEMI+MT as compared to the CER test. Finally, Figure 4 (panel C) shows that there was significant group, condition, and interaction effects in the COX (calculated as a percentage variation

from rest level). Specifically, the post-hoc analysis discovered that in the CTL group this parameter was higher in the PEMI+MT than in the CER, the PEMI, and the MT sessions. Statistics also found out that the CER+MT caused a higher COX level than the CER test. Finally, COX was more elevated in the PEMI+MT session of the CTL with respect to all sessions of the DM2 group.

2.1.7 Discussion

The main purpose of the experiment 1 was to assess whether the stimulation of the metaboreflex during a mental task was able to impair the COX in a group of patients suffering from DM2. In order to achieve this, the PEMI method was employed to trigger the metaboreflex as it allows to stimulate the sympathetic activity without any motor cortex activation. Thus, the increase in cerebral activity is supposed to be related only to the MT. That is, during the metaboreflex obtained with PEMI method the sympathetic tone is increased at a level similar to what experienced during exercise, but without any contribution of the central command (Crisafulli et al. 2015, Crisafulli 2017, Nobrega et al. 2014). This rules out any interference due to motor cortex activity on COX.

We found that, when MT was superimposed on the PEMI-induced metaboreflex activation, patients with DM2 could not increase COX to the same extent reached by the CTL group. In detail, looking at Figure 4 (panel C) it can be gleaned that during the session of MT alone (i.e. without PEMI), COX raised to a similar extent with respect to baseline in both groups (101.30±1.64% vs. 101.38±1.93% increase for the DM2 and the CTL group respectively). This increment in COX was likely due to the enhanced brain activity imposed by MT, which increased brain metabolism so elevating CBF and COX (Yaffe et al. 2004). We obtained similar results in a recent research with the same experimental setting (Doneddu et al. 2020). However, it can be also observed that the two groups behaved differently during the PEMI+MT test. Specifically, during the PEMI+MT test COX increased with respect to baseline in both groups in study, but in the CTL group the COX increment with respect to baseline was in the order of 4-5% (104.23±2.51%), while in the DMS group was lower (101.13±1.08%). This difference was statistically significant and demonstrates that adding the PEMI manoeuvre to the MT increased COX in normal individuals, but not in patients suffering from DM2. This supports the hypothesis that individuals with DM2 were not able to increase COX to the same extent as CTL subjects in this setting.

Another findings was that in the CTL group COX was higher during the PEMI+MT test than during all the sessions of the DM2 group, thereby strengthening the concept that healthy individuals have greater capacity to increase COX with respect to patients with DM2.

The COX increments obtained by the PEMI+MT test was likely the consequence of the combination of the slight COX increments caused by the PEMI manoeuvre and by MT. Actually, the possibility that PEMI induces an increase in COX has been already demonstrated and it was attributed to MAP elevation, although the exact physiological mechanism(s) is still unclear (Doneddu et al. 2020, Ogoh et al. 2019). Similarly, also the MT test can induce an increment in COX, which is likely the consequence of the enhanced brain metabolism due to brain activation. Of note, during the PEMI+MT test the DM2 group did not increase COX notwithstanding the MAP increment. It is well known that blood pressure is one of the key regulators of CBF and that increasing MAP results in CBF increments (Lucas et al. 2010, Willie et al. 2014). Thus, it may be expected that in the DM2 group the MAP increment that occurred during the PEMI+MT test would have led to higher COX levels in comparison with the other protocol conditions, but this was not the case. Hence, it seems as though in DM2 patients some phenomenon prevented the increase in CBF and COX notwithstanding the elevated MAP.

Several phenomena may explain the reduced capacity to increase COX in patients with DM2. One could be an impaired cerebral vascular reactivity. Some clues have been recently provided in support to the hypothesis that the capacity to vasodilate the cerebral circulation in response to physiological stimuli, such as hypercapnia, is impaired in individuals suffering from metabolic disorders such as MS and DM2. What causes this impaired vascular response has not be completely elucidated yet. One proposed mechanism is a low level of nitric oxide availability and a reduced endothelium-dependent vasodilation. However, the phenomenon is still elusive, likely multi-factorial, and further research is needed to better elucidate underlying mechanisms (Birdsill et al. 2013, Kim et al. 2015). Another potential mechanism explaining the impaired COX is that DM2 patients were not able to maintain SV and CO at the proper level, thereby challenging cerebral circulation. Actually, already at rest and throughout all the experimental sessions, the DM2 showed on average lower SV and CO values in comparison to the CTL group.

A further mechanism that could be involved in the impaired COX was an elevated SNS drive in DM2 patients. The cerebrovasculature is extensively innervated by adrenergic fibres which can potentially constrict brain circulation (Willie et al. 2014). However, the PEP behaviour does not support any exaggerated sympathetic drive as this parameter was similar between CTL and DM2 subjects; furthermore, it shortened to a similar extent in both groups during the protocol. PEP is the time spent by the left ventricle to develop the amount of pressure necessary to overcome aortic pressure and is inversely related to sympathetic activity, but it is not influenced by parasympathetic tone (Michael et al. 2017). The quite similar PEP response between groups suggests that in patients

with DM2 there was no exaggerated sympathetic activation. It is however to be highlighted that already at rest and throughout all the phases in the protocol, the DM2 patients showed a higher HR compared to the CTL group. Moreover, SVR levels were always more elevated in the DM2 in comparison to the CTL group. It is well known that HR and arteriolar tone are directly related to SNS activity (Joyner 2016). Both phenomena (i.e. elevated HR and SVR) suggest that SNS tone was higher in the DM2 patients as compared to the CTL, notwithstanding the similar PEP. A potential explanation for these conflicting results could be that, in the DM2 group, SNS was already more activated at rest to support a failing circulation, as testified by the lower SV levels. The elevated SNS tone already at rest may have reduced the possibility of highlighting any increases in SNS due to PEMI and/or MT engagement. Only direct measurement of SNS activity can solve these contradictory findings.

Whatever the causes (impaired capacity for cerebral vasodilation, reduced capacity to increase SV, and increased SNS activity), results demonstrate that patients with DM2 were unable to properly increase COX when a MT superimposed the metaboreflex elicited by PEMI. This finding suggests that patients with DM2 suffered from a sort of impairment in cerebral flow which resembles what recently found in patients with MS, who could not increase COX to the same extent as normal controls (Doneddu et al. 2020). It should be considered that MS often precedes DM2 and that DM2 patients may experience SNS dysfunction even worse than subjects with MS (Eckel et al. 2005, Istenes et al. 2014).

It has been demonstrated that the combination of a mental and physical challenges results in an exacerbation of stress hormones responses than a single challenge alone (Webb et al. 2017). This is of importance as individuals are often subjected to a combination of stressors during exercise. In particular, mental stress could represent an important stressor for the cardiovascular system (Rozanski et al. 1988, Wasmund et al. 2002). Moreover, COX may be a limiting factor for exercise capacity in patients suffering from DM2. Although the brain represents only ~2% of the total body mass, it consumes up to ~20% of the available oxygen at rest and precise control of CBF is imperative, especially during exercise when its activity increases (Secher et al. 2008). Adherence to physical exercise programs is poor in patients with DM2 (Poitras et al. 2018); it was proposed that the incapacity to increase COX could provide a potential physio-pathological basis for this poor predisposition as the presence of reduced O2 supply clearly may compromise the willingness to sustain a given level of effort (Kim et al. 2015). Similarly, this possibility has been proposed also for patients with MS (Doneddu et al. 2020), this also considering that MS impairs cognitive performance when an attentional interference task was added to PEMI (Guicciardi et al. 2020).

Results of the present investigation support the hypothesis that the combination of MT and cardiovascular reflex impairs COX, thereby providing a potential physio-pathological basis for poor predisposition to exercise of these patients.

From an hemodynamic point of view, the cardiovascular response showed by DM2 patients during PEMI was in good agreement with what reported in previous studies employing a similar experimental setting (Roberto et al. 2019). Specifically, it was reported that DM2 patients had an exaggerated vasoconstriction (i.e. SVR increase) in response to PEMI after handgrip, while SV and CO were blunted. This was the consequence of a reduced capacity to enhance myocardial performance. Therefore, in these patients, the target blood pressure was achieved mainly by an increase in SVR rather than by a CO-mediated response (Roberto et al. 2019). In the present investigation, VER (a measure of myocardial performance) was always lower in DM2 patients as compared to CTL, so confirming that a reduced systolic functions were present in DM2 and this hampered SV. This phenomenon may have triggered compensatory mechanisms leading to SNS activation already at rest and in turn increasing arteriolar constriction. This is a type of response that is also observed in a number of other cardiovascular diseases (Piepoli and Crisafulli 2014, Crisafulli 2017), thereby supporting the point of view that DM2 can be considered a cardiovascular disease (Candido et al. 2003).

2.2 ***Experiment 2***

2.2.1 Study Population.

We recruited a total of twenty-eight individuals; 16 aged 18-30 years (YG) and 16 aged ≥50 years (AG).

We enrolled participants without impairment in cognitive function, determined by Montreal Cognitive Assessment (MoCa) (Nasreddine, 2005) Score ≥ 26 at the day of the experimental session. For both groups, exclusion criteria included diagnosis of significant neurological or psychiatric diseases, relevant for brain function or CBF (e.g. stroke, dementias), a history of brain surgery. Smokers and participants using B-blockers, sympathomimetics and/or tricyclic antidepressants were also excluded due to their potential impact on cerebral perfusion. Participants were requested to abstain from caffeinated beverages, alcohol, and heavy exercise 12 h prior to the measurements. Written informed consent was obtained from all participants. Ethical approval was

obtained from the local ethics committee and the study was conducted according to the principles of the Declaration of Helsinki (2013).

2.2.2 Experimental Design

The study comprised a single visit to the Geriatrics Department of the Radboudumc, Nijmegen, the Netherlands. After arrival, participants seated at rest in a temperature-controlled (20-22°C) room for 10 minutes to ensure an accurate baseline measurement. During 7 minute of rest, middle cerebral artery blood flow velocity (MCAv) and BP were assessed. All participants performed in the following order: 1) 5 min standing (STAND), 2) 5 min walking at 15% (WALK15) of maximum heart rate (HR_{max}), 3) 5 min walking at 30% of HR_{max} (WALK30). During all procedures, we continuously BP, HR, and MCAv. Between all sessions, participants had a 7-minutes seated rest (SIT) to allow cerebral perfusion to return to baseline levels and to have a similar starting position for each daily life condition.

2.2.3 Experimental Daily Life Activities

Figure 2.1 schematises the various sessions of the study protocol:

STAND. Subjects stand in the upright position for 5-minutes.

WALK15. Participants performed walking exercise on the spot for 5-minutes (as practical limitations of the TCD (Trans-cranial Doppler) equipment prohibit free walking activities). During walking, HR was monitored and individuals were instructed to change their pace to increase heart rate to match 15% of the predicted HR_{max} (208-0.7*age). Participants were coached to perform the walking speed in order to reach the predicted HR, which was kept unvaried for the whole walking session.

WALK30. The same walking protocol used for *WALK15* was performed. Individuals were instructed to reach 30% of the predicted HR_{max}, and to keep the walking pace steady.

2.2.4 Hemodynamic and cerebral blood flow assessment

Before the start of the study protocol the resting blood pressure was determined using an automatic blood pressure cuff (Brand name, Country). Cerebral blood flow velocity (CBFv) in the middle cerebral arteries was measured using two 2-MHz transcranial Doppler probes (TCD, Multi-Dop, Compumedics DWL, Singen, Germany). The TCD monitored the changes in cerebral blood flow velocity in the middle cerebral artery during the measurement on both the right and left side, accessed through the temporal bone (‘’temporal window’’). This was done under the assumption

that the vessel diameter stays constant during the measurement, so that changes in cerebral blood flow velocity would actually represent changes in cerebral blood flow(Claassen et al. 2007). Continuous blood pressure was measured using photoplethysmography (Finapres Medical Systems, Amsterdam, the Netherlands). A blood pressure cuff was placed on the middle finger, allowing for monitoring of the participant throughout the assessment. To correct for the influence of blood pressure on MCAv during daily life activities, cerebrovascular conductance was calculated by dividing the MCAv by the blood pressure.

HR was measured by means of a monitor heart rate (Polar RS400, Polar, Oy, Kempele, Finland) band placed at heart height around the chest.

All physiological data measurements were continuously acquired at 50 Hz using an analog-to-digital convertor (PowerLabML880; ADInstruments, Colorado Springs, CO) and displayed in real time on a computer with commercially available software (Acknowledge 2.0, BIOPAC systems, USA).

2.2.5 Data Analysis and Calculation

Data were extracted in means per minute, which allowed for calculation of the average across the last 3 minutes, and analysed using IBM SPSS Statistics 22.0 (SPSS, Inc, Chicago, IL). To correct for the potential impact of a change in CBFv and BP to correct for changing posture, relative values as percentage changes from standing were reported. All data are presented as mean±SEM for continuous variables, as number (percentage) for categorical variables and as median (interquartile range) for skewed distributed data, unless stated otherwise. The statistical analysis of the data were performed with a general linear mixed model. The general linear mixed model was used to examine the differences between the age groups, the effect of the daily life activities and also whether groups (young and old) demonstrated different changes in the study parameters (interaction-effect: group*condition). For all these analyses a significance level of α=0.05 was used.

2.2.6 Results

2.2.6.1 Anthropometrics

In two participants we were unable to assess cerebrovascular blood flow due to technical difficulties, leaving 15 YG (24.47 ± 2.22 years) and 15 AG (64.36 ± 5.86 years) participants with valid data for analysis. Subject characteristics, including age, gender, BMI and MoCA score of the participants, can be found in Table 2.1.

2.2.6.2 Effect of standing and walking to correct for sitting

CBFv significantly changed during the conditions (P=0.01), with a lower CBFv during standing compared to WALK15 and WALK30 (Figure 2.1A, 2.1B and 2.1C). When evaluating BP data, a significant effect of the conditions was observed, with higher BP during WALK15 and WALK30 (P<0.01) (Figure 1C and 1D). Conductance showed a significant decrease during standing (P<0.01), whereas no difference was found during WALK15 and WALK30 (Figure 2.1G, 2.1H and 2.1I).

When comparing YG and AG subjects, YG had a significant higher CBFv compared to AG (P<0.01). Moreover, AG showed a larger increase in CBFv during WALK15 compared to YG participants (P=0.01). BP did not differ significantly between YG and AG during the conditions (P=0.29, P=0.26 and P=0.27). A significant interaction effect was observed in the AG (P=0.01) when compared to the YG, who increased BP to a larger extent during WALK15 and WALK30 (P<0.01). In term of cerebrovascular conductance, AG had lower values throughout the conditions (P<0.01).

2.2.6.3 Effect of walking to correct for standing

CBFv significantly increased during walking compared to stand (P=0.01), with a higher response in CBFv during WALK15 (P=0.02, Figure 2.2A). BP showed overall a significant increase during the conditions (WALK15 and WALK30, respectively) (P<0.01), with a higher response in the AG when compared to the YG (P=0.05, Figure 2.2B).

Finally, no interactions between groups and conditions were observed for cerebrovascular conductance (Figure 2.2C).

2.2.7 Discussion

The first purpose of experiment 2 was to characterise the impact of daily life activities (i.e sitting, standing, walking) on the cerebral blood flow in healthy (YG and AG) individuals. We found that daily life activities, such as walking at 15% and 30% of HRR lead to an immediate increase in CBFv of around 3-7%. Although these changes are relatively modest, our work highlights that even common daily life activities can have a marked impact on CBFv. The second purpose of this study was to find out whether changes in CBF were different between YG and AG during DLA. We do not find any significant difference between YG and AG, however AG showed a larger increase both in CBFv and BP during the walking period. Given the importance of cerebral perfusion in cognitive decline, this finding may help to better understand the potential clinical importance of engagement in daily life activities. Several studies have investigated the impact of exercise at low, moderate,

high and maximal intensity on CBFv, and reported a change in CBFv between -10 and 40% (Fisher et al. 2013, Braz et al. 2016, Smith and Ainslie 2017). Braz et al. combined data from these studies to look at the mean increase in CBFv, and described an increase around 10% during low and maximum intensity, 15-20% for moderate intensity and around 20% for high intensity (Braz et al. 2016). Thus, when compared to our data, the response of CBFv during low intensity activities was similar to that described in previous studies. This increase in CBFv could be explained by several mechanisms that contribute to regulate the CBF, such as cerebrovascular reactivity and other vasoactive adjustments mainly driven by changes in arterial blood gases, blood pressure, cerebral metabolism and neurogenic innervation (Braz et al. 2016). During exercise, cerebral blood flow and cerebral oxygenation increase due to cerebral activation, as well as arterial blood pressure, heart rate, cardiac output and sympathetic activity (Nobrega et al. 2014). Likewise, arterial blood gases such as arterial carbon dioxide tension (PaCO2) increases or decreases based on the level of alveolar ventilation determined by the intensity of the exercise. For instance, performance of exercise at low (25% Wmax) to moderate intensity (50% Wmax) is characterized by an increase in CBFv (Thomas et al. 1989). In contrary, at higher intensity (>50-80% Wmax) CBFv tends to a plateau or shows a progressive decrease, depending on the level of PaCO2 (Smith and Ainslie 2017). In our study we did not measure PaCO2 and therefore we cannot exclude a potential impact of changes in PaCO2 on our results. In contrast to walking, during standing a significant drop in CBFv and conductance was observed. These changes were not accompanied by a drop in blood pressure, as we only included the last three minutes of standing, which allowed BP to stabilize. Over the first minute of standing often a drop in BP is observed, caused by transient orthostatic hypotension (Novak et al. 1998), this is because the vascular adjustments initiated by arterial pressure are not instantaneous and take several seconds to take effect. However, rapid variations in arterial pressure as from sit to standing position are not compensated for by autoregulation and produce changes in CBF (Florence et al. 1992), this phenomenon may also explain the response in CBFv and BP. When evaluating the effect of walking to correct for changing in posture we found a significant increase in CBFv. In detail during WALK15 an increase around 3% was detected, whereas during WALK30 was found around 2.5 %. This suggest that even very low intensity activity can affect CBFv, despite the magnitude of the effort. Recent studies reported that prolonged uninterrupted sitting causes a decrease in CBFv, whereas short duration regular walking breaks has a positive effect on cerebral perfusion preventing cerebral decline (Carter et al. 2018). Thus, our data are in line with these previous studies: Hartman et al. found that increases in daily life activity

over 16 weeks also increased CBFv and may also have important clinical implication by promoting active break strategies for those who engage in long period of sitting (Hartman et. al 2020).

The second purpose of experiment 2 was to find out whether changes in CBF were different between YG and AG during daily life activities. Despite the fact CBFv was consistently lower in older participants, no significant differences were observed in the response to the activities between groups, with the only exception during walking where AG showed a larger increase in CBFv in comparison to YG. In detail, statistics revealed that older individuals had a larger increase in CBFv during WALK15, which can be explained by the parallel larger increase in BP. In fact, AG showed a higher response in BP throughout all the activities, with higher values during WALK15. Thus, according to our results low intensity activities had a larger impact in BP response in AG when compared to young. It looks like that AG may have higher fluctuation in BP either when sitting or during exercise, or probably they just performed the activities at higher intensity compared to YG. Another potential explanation for this could be the change in PaCO2 caused by the different ventilation during the activities, which may have an influence on both CBFv and BP changes, suggesting the cerebral autoregulation mechanism could be altered in more elderly individuals (Paulson et al. 1990). However, we did not measure PaCO2 so further measurements should be take into account to support the previous statement. As mentioned before when compared to YG, the AG had consistently lower CBFv in all activities. This finding is in line with previous assessments in population of a similar age using transcranial Doppler ultrasonography (Ainslie et al. 2008). A study conducted by Ainslie et al. found an age related decline in CBFv of -0.76 ± 0.04 cm/s/year, when comparing sedentary elderly and endurance-trained young men. Translating this to our population, this suggests a difference of 30 cm/s between our YG and A group. We however observed a difference of 12 cm/s, probably because our population was neither sedentary nor endurance trained, making the difference smaller between both groups. Moreover, conductance was found constantly lower in the AG throughout activities, which can also be ascribed to the age-related circulatory alterations such as endothelial disfunction and attenuated vasodilation, already described in previous studies (Fisher et al. 2013, Fluck et al. 2014, Tarumi and Zhang 2018). This implies that age should be taken into account when interpreting data of CBF and that ageing per se may challenge mechanisms regulating brain perfusion during day life activities.

2.3 Experiment 3

2.3.1 Study Population

Forty healthy men aged 31-59 years old with no history of cardiovascular disease (CVD) were involved. Exclusion criteria were: a history of CVD, history of DM, currently using cardiac medication for heart rate, blood pressure or cholesterol, Raynaud's syndrome. Local ethical approval from the Liverpool John Moores University was sought and gained. Informed consent was obtained and formally documented. Participants completed a health questionnaire, including medical history and CVD related lifestyle risk factors.

2.3.2 Experimental Design

The day of the measurement participants body weight and height were measured, and they were instructed to lie on a bed in a quiet, light and temperature controlled room. A finometer (Finapres Medical Systems, Amsterdam, The Netherlands) was used to measure beat-to-beat blood pressure and the resting blood pressure was measured with an automated sphygmomanometer (Dinamap Procare 100, GE Medical Systems Ltd., Buckinghamshire, UK). Participants laid supine for at least 5 minutes before they underwent the cold pressure test (CPT). The Cold Pressor Test is a sympathetic nervous system stimulus consisting of 1 minute baseline, 3 minutes with the left hand submerged in cold water (~4°C). During the CPT, carotid artery diameter and blood flow velocity were measured continuously using ultrasound sonography (Terason 3300, Terason Labs, Burlington, Massachusetts, USA).

2.3.3 Measurements

The BP was measured with an automated sphygmomanometer (Skirton et al. 2011) on the left arm while the participant was laying supine. This measure was used to determine the resting BP and to calibrate the beat-to-beat BP values. The finometer cuff was attached on the second phalanx of the right index or middle finger. The finometer was calibrated to the height of the heart and was allowed to auto-calibrate for 2 minutes. This has previously been demonstrated to be a reliable and reproducible measure of beat-to-beat blood pressure monitoring (Waldron et al. 2018).

2.3.4 Cold pressor test

During the CPT, the left hand was immersed in a bucket of cold water (~4°C). The water temperature was measured with a digital thermometer (Quartz digi-thermo, Fischer scientific, Loughborough, UK) and controlled by adding crushed ice to maintain a stable water temperature.

The participant was asked to position themselves close to the left edge of the bed, to ensure the hand could easily move into the water without significant movement of the neck. This enabled assessment of the carotid artery. After a 1 minute baseline diameter recording, participants were instructed to place their hand in the ice water for 3 minutes. They were instructed not to speak, and to breathe normally during the ultrasound assessment of the carotid artery in order to prevent hyperventilation (van Mil et al. 2017).

2.3.5 Carotid Artery Diameter

The left common carotid artery diameter was assessed using ultrasound sonography (Terason 3300, Terason Labs, Burlington, Massachusetts, USA). Using a longitudinal view of the artery, the carotid bulb was identified as an anatomical landmark to standardise approximate scanning area between individuals. The common carotid artery, proximal from the carotid bulb, was identified and image was optimised so that the artery walls were clearly defined (figure 3.1). Doppler velocity assessments were also recorded at the lowest possible insonation angle (always <60°). The carotid artery diameter was calculated with edge detection software (Potter et al. 2007). On-screen calibration points were selected with the calibration tool which the software calculated the pixel-to-centimetre ratio. Calibration points were used for the diameter and the pulse wave velocity. A rectangle containing the largest straight artery segment was selected as the Region of Interest (ROI), ensuring that the vessel walls were in focus. The software marked the vessel walls within the ROI with lines and calculated the number of pixels in each vertical column between the lines. From the pixel distance the software calculated the lumen diameter in centimetres.

2.3.6 Carotid Artery Response (CAR%)

The CAR% is the relative change in carotid artery diameter above or below baseline expressed as a percentage. The average diameter during the 1 minute baseline measurement was calculated and set as the baseline value. Subsequently, the diameter of the carotid artery was measured during the CPT, and averaged over 10 second periods, resulting in 24 periods. The average, maximum and minimum percentages, were calculated. If the average percentage change was an increase in diameter (dilation), the CAR% is expressed as the maximum percentage. Conversely, if the average percentage change was a decrease in diameter (constriction), the CAR% is expressed as the minimum percentage (van Mil et al. 2017, Fluck et al. 2017).

2.3.7 Blood pressure

The beat-to-beat BP data was processed in the same way as the CAR% calculation. Beat-to-beat BP was measured and then MAP calculated. Baseline was the average MAP during the 1 minute. Next, the average MAP during the CPT is calculated for each 10 second period, matching the epochs for diameter as described earlier. The SBP and DBP measured with the sphygmomanometer is used to calculate the MAP before testing. The beat-to-beat BP data was calibrated using assessment of resting BP using an automated sphygmomanometer (Dynamap) placed around the left arm and performed twice (with a 5-minute rest period in between). The maximum change in BP was expressed as the maximum increase (ΔMAP) and maximum percent increase in MAP (relative ΔMAP) compared to baseline during the CPT.

2.3.8 Data analysis and Calculation

All data were presented as mean ± SD. Statistical analysis was performed using IBM SPSS Statistics 25 (IBM SPSS; IBM Corp., Armonk, New York, USA). Pearson correlations were employed to examine the relation between baseline MAP and the change in BP during the CPT (ΔMAP) versus the CAR% (i.e. relative change in diameter compared to baseline), whilst we also examined the relation between the timing of the peak responses in BP versus CAR%. Participants were divided in tertiles based on the relative change in BP during the CPT: low (<15%), medium (15-30%) and high (>30%). One-way ANOVA was used to examine difference between groups in general characteristics including age, BMI and cardiovascular risk and CAR%. Tukey post-hoc analysis was performed to examine which groups differed from each other. Statistical significance was at $p<0.05$.

2.3.9 Results

In response to the CPT, diameter immediately changed and demonstrated a gradual increase with an average peak at 92±43 seconds, which was followed by a gradual decline (Figure 3.2A). The mean CAR% was 4.4%±5.4, with six participants demonstrating a constriction of the carotid artery during the CPT (ranging from -7.6 to –0.74%). During the CPT, MAP began to increase within 30s, followed by a gradual increase that peaked at 112±38 seconds (Figure 3.2A). The timing of the peak MAP (112±38s) was significantly later than the peak in diameter (92±43s, difference in peak 20±5s, Wilcoxon-test; P=0.04). There was no significant correlation between peak CAR% and peak MAP (R=0.03 P=0.29), nor between the timing of the CAR% and MAP (R=0.03 P=0.30). After dividing the group into tertiles (based on the relative increase in BP), no significant differences were found

between groups in subject characteristics (e.g. age, weight, BMI, MAP and family history), baseline diameter or CAR% (Table 3.1). No differences were found between groups when, individuals were divided into tertiles based on absolute BP responses (data not shown). There was no correlation between the relative increase in blood pressure and CAR% ($r=0.27$ $p=0.09$). Based on the distinct vasomotor responses during the CPT, we compared groups with carotid artery dilation (n=34) versus constriction (n=6). Nevertheless, baseline diameter and CAR% were similar (Table 3.2). Importantly, participants who demonstrated carotid artery constriction revealed a similar increase in BP compared to individuals with carotid dilation (Table 3.3).

2.3.10 Discussion

The primary aim of experiment 3 was to understand the relationship between changes in blood pressure and carotid artery diameter during the CPT. We found that the start of dilation and the timing of the peak carotid artery diameter response preceded blood pressure changes during the CPT. Moreover, we found no differences in baseline characteristics including age, weight, BMI or in the magnitude of carotid artery dilation when comparing groups based on the magnitude of BP increase. This finding is supported by the lack of correlation between the relative changes in carotid artery diameter and blood pressure during the CPT. Finally, individuals who demonstrated carotid artery vasoconstriction also demonstrated a comparable increase in BP during sympathetic stimulation compared to those with vasodilation. Taken together, experiment 3 suggests that the characteristic increase in blood pressure during sympathetic stimulation may not directly relate to carotid artery vasomotion in healthy middle-aged men.

The CPT is a frequently used procedure to activate the sympathetic nervous system in humans. As expected, and in line with several previous studies, BP gradually increased after a period of 20-30 seconds. The increase in BP is most likely the result of (nor)adrenaline release, mediating a vasoconstriction response in peripheral arteries that cause an increase in total peripheral resistance. (Wirch et al. 2006) We found that the timing of the start of carotid artery dilation, but also the timing of the peak diameter change, significantly preceded the BP response. Thus, contrary to our hypothesis, carotid artery response is not directly linked or driven by the increase in blood pressure response during the CPT. Furthermore, we found no relation between the degree of BP increase and the CAR% during the CPT. However, it should be noted that the lack of correlation may relate to the presence of confounding factors influencing vascular tone. Closely controlling for factors potentially affecting endothelial function (e.g. drugs, supplements, behavioural aspects) at least partly prevented such impacts. A final strong argument against a key role for BP in mediating the

carotid artery vasomotor response during the CPT is the presence of vasoconstriction in some individuals. Moreover, a significant increase in BP was found in these individuals, which did not differ from the BP response found in subjects with carotid artery dilation. Despite the absence of a relation between the diameter and BP response, both responses seem strongly related to sympathetic stimulation. For instance, a previous study found that muscle sympathetic nerve activity bursts are associated with concomitant increases in BP and peripheral conduit artery diameter responses (Fairfax et al. 2013). Furthermore, catecholamine-release during sympathetic stimulation seem directly related to carotid artery responses, whilst catecholamines may also be responsible for the increased peripheral artery resistance and BP changes (Gordan 2015). Differences in sensitivity of receptors or mechanisms contributing to vasomotion between central (i.e. carotid) and peripheral arteries may explain the difference in timing of the BP and diameter responses. Nonetheless, given their dependence on catecholamines (Currie et al. 2012), we expected a relation between the magnitude of BP and diameter response. One potential explanation for the lack of relation is that catecholamine-release is less strongly related to vascular responses than anticipated. Indeed, Cummings et al. found adrenalectomised participants do not demonstrate an increase in adrenaline, noradrenaline or dopamine during the CPT, despite the presence of an increase in BP of comparable magnitude as in healthy individuals. Therefore, peripheral artery responses (and therefore blood pressure) to the CPT may be independent of catecholamine release (Cummings et al. 1983), whilst catecholamines may be important for carotid artery diameter responses. At least, our data suggests no direct link between BP per se and carotid artery diameter response to the CPT in healthy individuals, despite both parameters change markedly in response to CPT. Based on the important role of BP during the CPT, and the possible link with vasomotion, we recommend performing beat-by-beat BP measurements when examining the CAR. It has to be consider is that structural properties of the artery may influence the dilator response. A previous study demonstrated a negative correlation between baseline carotid diameter and CAR% in non-diseased average risk, high risk, and coronary artery disease patients, but not with the carotid artery intima-media wall thickness (IMT) (Rubenfire et al.2000). In contrast, Van Mil et al. reported no correlation between the baseline carotid diameter or IMT and CAR% in healthy people (van Mil, Hartman et al. 2017), whilst also others found no correlation between coronary artery baseline diameter and dilation response (Vita et al. 1990). In our experiment, we found a significant, but weak, inverse correlation between the baseline carotid diameter and the CAR%, implying that a smaller baseline diameter correlates with a larger CAR%. This finding is in line with several previous studies examining peripheral arteries, where a smaller brachial or femoral artery is related to a larger dilation in

response to increases in shear stress (Padilla et al. 2011, Thijssen et al.2008). The correlation between diameter and CAR% in our study, whilst largely absent in previous work, may relate to the inclusion of healthy individuals only. In detail, previous work in peripheral arteries also found a weaker or non-existing correlation between baseline diameter and dilator responses in older and diseased populations. This may be explained by the impact of older age and/or cardiovascular risk factors in those groups that affect both baseline diameter and dilator response, consequently affecting the (weak) inverse relation between both parameters in healthy young individuals. Finally, our observation suggests that structural characteristics of the artery wall should be considered when examining the CAR% responses, but unlikely affect or interfere with the blood pressure increase (and subsequent diameter response) during the CPT.

CONCLUSIONS

The results of experiment 1 support the hypothesis that subjects suffering from DM2 do not properly enhance COX when a mental task superimposed the metaboreflex obtained by PEMI. Thus our data support that patients with metabolic disorders experience reduction in cerebral oxygenation as well as sympathetic dysfunction in response to exercise, with exaggerated and paradoxical vasoconstriction at brain level. Further studies with a larger sample size are warranted to confirm the phenomenon and to clarify mechanism behind this phenomenon. Further research is also warranted to clarify whether the described COX impairment is present also during real exercise. Finally, results confirm that in DM2 patients' hemodynamic regulation is altered during the PEMI-induced metaboreflex as in these patients the target BP is achieved by increasing SVR rather than by increasing CO. This fact would provide the physio-pathological basis of the bad exercise predisposition often observed in these patients.

Experiment 2

In experiment 2 our data support that even low-to-moderate intensity daily life activities significantly alter the cerebral perfusion, whilst these changes in cerebral perfusion are largely comparable between AG and YG. We found that DLA, which are common in daily living (i.e sitting, standing and walking), lead to an immediate increase in CBFv measured as the middle cerebral artery velocity (MCAv) both in YG and AG. Although these changes are relatively modest, this study highlights that even common DLA can have a marked impact on CBFv and they may link to health benefits of regular daily life physical activities. Further research is warranted to

clarify whether the described finding is present also during exercise at different intensity levels. Furthermore, we do not find any significant difference between YG and AG, despite AG showed a larger increase both in CBFv and BP during the walking period. Thus, given the importance of cerebral perfusion in cognitive decline, this finding may help to better understand the potential clinical importance of engagement during DLA in healthy individuals.

Lastly, experiment 3 highlight that carotid artery diameter changes during the CPT may not be related to the characteristic increase in blood pressure. The start and peak of the diameter precedes that of the BP, whilst no correlation is present between the magnitude of the blood pressure response and CAR%. Moreover, even individuals who present carotid artery vasoconstriction demonstrate an increase in BP, making it unlikely that the BP rise should be regarded as the dilator stimulus. Nonetheless, the change in BP during the CAR% may still be relevant, especially to understand the link to the sympathetic nervous system. This study suggests the CAR% provides relevant information, independent of the increase in blood pressure during the CPT. In conclusion, CAR% may represent a valuable, simple, easy applicable and non-invasive technique to assess the sympathetically-induced elevation in blood pressure and carotid artery diameter responses in healthy middle-aged individuals.

References

1. Aaslid R. Cerebral hemodynamics. In: Transcranial Doppler, edited by Newell DW and Aaslid R. New York: Raven, 1992, p. 49–55.
2. Ainslie PN, Cotter JD, George KP, Lucas S, Murrell C, Shave R, Thomas KN,Williams MJ, Atkinson G. Elevation in cerebral blood flow velocity with aerobic fitness throughout healthy human ageing. J Physiol, 2008, 586, 4005–4010.
3. Ainslie PN, Poulin MJ. Ventilatory, cerebrovascular, and cardiovascular interactions in acute hypoxia: regulation by carbon dioxide. J Appl Physiol , 2004, 97: 149–159.
4. Alam M, Smirk FH. Observations in man on a pulse-accelerating reflex from the voluntary muscles of the legs. J Physiol. 1938 Mar 14;92(2):167-77.
5. Alam M, Smirk FH. Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. J Physiol. 1937 Jun 3;89(4):372-83.
6. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart JC, James WP, Loria CM, Smith SC Jr; International Diabetes Federation Task Force on Epidemiology and Prevention; Hational Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; International Association for the Study of Obesity. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009 Oct 20;120(16):1640-5.
7. Amann M, Blain GM, Proctor LT, Sebranek JJ, Pegelow DF, Dempsey JA. Implications of group III and IV muscle afferents for high-intensity endurance exercise performance in humans. J Physiol. 2011; 589: 5299-309.
8. American Diabetes Association. Economic costs of diabetes in the U.S. in 2017. Diabetes Care 2018;41:917–928
9. Andreani CM, Hill JM, Kaufman MP. Responses of group III and IV muscle afferents to dynamic exercise. J Appl Physiol (1985). 1997 Jun;82(6):1811-7.
10. Asahara R, Matsukawa K, Ishii K, Liang N, Endo K. The prefrontal oxygenation and ventilatory responses at start of one-legged cycling exercise have relation to central command. J Appl Physiol (1985). 2016 Nov 1;121(5):1115-1126.
11. Atkinson JL, Anderson RE, Sundt TM Jr. The effect of carbon dioxide on the diameter of brain capillaries. Brain Res , 1990, 517: 333–340.
12. Baker LD, Frank LL, Foster-Schubert K, Green PS, Wilkinson CW, McTiernan A, Plymate SR, Fishel MA, Watson GS, Cholerton BA, Duncan GE, Mehta PD, Craft S. Effects of aerobic exercise on mild cognitive impairment: a controlled trial. Arch Neurol. 2010 Jan;67(1):71-9.
13. Beason-Held LL, Moghekar A, Zonderman AB, Kraut MA, Resnick SM. Longitudinal changes in cerebral blood flow in the older hypertensive brain. Stroke, 2007, 38, 1766–1773.
14. Bernstein DP. A new stroke volume equation for thoracic electrical bioimpedance: theory and rationale. Crit Care Med. 1986 Oct;14(10):904-9.
15. 14. Birdsill AC, Carlsson CM, Willette AA, Okonkwo OC, Johnson SC, Xu G, Oh JM, Gallagher CL, Koscik RL, Jonaitis EM, Hermann BP, LaRue A, Rowley HA, Asthana S,

Sager MA, Bendlin BB. Low cerebral blood flow is associated with lower memory function in metabolic syndrome. Obesity (Silver Spring). 2013 Jul;21(7):1313-20.
16. Brian JE Jr, Faraci FM, Heistad DD. Recent insights into the regulation of cerebral circulation. Clin Exp Pharmacol Physiol , 1996, 23: 449–457.
17. Busija DW, Heistad DD. Factors involved in the physiological regulation of the cerebral circulation. Rev Physiol Biochem Pharmacol, 1984, 101:161–211.
18. Candido R, Srivastava P, Cooper ME, Burrell LM. Diabetes mellitus: a cardiovascular disease. Curr Opin Investig Drugs. 2003 Sep;4(9):1088-94.
19. Carnethon MR. Diabetes prevention in US ethnic minorities: role of the social environment. J Am Diet Assoc. 2008 Jun;108(6):942-4.
20. Claassen JA, Zhang R, Fu Q, Witkowski S, Levine BD. Transcranial Doppler estimation of cerebral blood flow and cerebrovascular conductance during modified rebreathing. J Appl Physiol (1985). 2007 Mar;102(3):870-7.
21. Coetsee C, Terblanche E. Cerebral oxygenation during cortical activation: the differential influence of three exercise training modalities. A randomized controlled trial. Eur J Appl Physiol. 2017 Aug;117(8):1617-1627.
22. Colcombe S, Kramer AF. Fitness effects on the cognitive function of older adults: a meta-analytic study. Psychol Sci. 2003 Mar;14(2):125-30.
23. Crisafulli A, Marongiu E, Ogho S. Cardiovascular reflexes activity and their interaction during exercise. Biomed Res Int, 2015; 2015:394183.
24. Crisafulli A, Melis F, Lai AC, Orrù V, Lai C, Concu A. Haemodynamics during a complete exercise induced atrioventricular block. Br J Sports Med. 2002 Feb;36(1):69-70.
25. Crisafulli A, Melis F, Orrù V, Lener R, Lai C, Concu A. Impedance cardiography for non-invasive assessment of systolic time intervals during exercise. Sports Medicine Training and Rehabilitation. 2001; 10, 13-27.
26. Crisafulli A, Milia R, Lobina A, Caddeo M, Tocco F, Concu A, Melis F. Haemodynamic effect of metaboreflex activation in men after running above and below the velocity of the anaerobic threshold. Exp Physiol. 2008 Apr;93(4):447-57.
27. Crisafulli A, Milia R, Vitelli S, Caddeo M, Tocco F, Melis F, Concu A (2009). Hemodynamic Responses To Metaboreflex Activation: Insights From Spinal Cord-Injured humans. Eur J Appl Physiol 106: 525-533.
28. Crisafulli A, Piepoli MF, Thijssen D, Bassareo PP. Editorial: Cardiovascular Adjustments and Adaptations to Exercise: From the Athlete to the Patient. Front Physiol. 2020 Feb 27;11:187.
29. Crisafulli A, Piras F, Filippi M, Piredda C, Chiappori P, Melis F, Milia R, Tocco F, Concu A. Role of heart rate and stroke volume during muscle metaboreflex-induced cardiac output increase: differences between activation during and after exercise. J Physiol Sci. 2011 Sep;61(5):385-94.
30. Crisafulli A, Salis E, Pittau G, Lorrai L, Tocco F, Melis F, Pagliaro P, Concu A. Modulation of cardiac contractility by muscle metaboreflex following efforts of different intensities in humans. Am J Physiol Heart Circ Physiol. 2006 Dec;291(6):H3035-42.
31. Crisafulli A, Scott AC, Wensel R, et al. Muscle metaboreflex induced increases in stroke volume. Med Sci Sports Exerc 2003;35: 221-8.
32. Crisafulli A, Tocco F, Milia R, Angius L, Pinna M, Olla S, Roberto S, Marongiu E, Porcu M, Concu A. Progressive improvement in hemodynamic response to muscle metaboreflex in heart transplant recipients. J Appl Physiol (1985). 2013 Feb;114(3):421-7.

33. Crisafulli A. The impact of cardiovascular diseases on cardiovascular regulation during exercise in humans: studies on metaboreflex activation elicited by the post-exercise muscle ischemia method. Curr Cardiol Rev. 2017, 13: 293–300.
34. D'Alecy LG, Rose CJ, Sellers SA. Sympathetic modulation of hypercapnic cerebral vasodilation in dogs. Circ Res, 1979, 45: 771–785.
35. De Leon MJ, George AE, Ferris SH, Christman DR, Fowler JS, Gentes CI, Brodie J, Reisberg B, Wolf AP. Positron emission tomography and computed tomography assessments of the aging human brain. J Comput Assist Tomogr, 1984, 8, 88–94.
36. Delaney EP, Greaney JL, Edwards DG, Rose WC, Fadel PJ, Farquhar WB. Exaggerated sympathetic and pressor responses to handgrip exercise in older hypertensive humans: role of the muscle metaboreflex. Am J Physiol Heart Circ Physiol. 2010 Nov;299(5):H1318-27.
37. Dupuy O, Gauthier CJ, Fraser SA, Desjardins-Crèpeau L, Desjardins M, Mekary S, Lesage F, Hoge RD, Pouliot P, Bherer L. Higher levels of cardiovascular fitness are associated with better executive function and prefrontal oxygenation in younger and older women. Front Hum Neurosci. 2015 Feb 18;9:66.
38. Doneddu A, Roberto S, Pinna V, Magnani S, Ghiani G, Sainas G, Mulliri G, Serra S, Kakhak SAH, Milia R, Lecis R, Guicciardi M, Crisafulli A. Effect of Combined Mental Task and Metaboreflex Activation on Hemodynamics and Cerebral Oxygenation in Patients With Metabolic Syndrome. Front Physiol, 2020, 11: 397.
39. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet, 2005, 365(9468): 1415-1428.
40. Edvinsson L, Krause DN. Cerebral Blood Flow and Metabolism. Philadelphia: Lippincott, Williams & Wilkins, 2002.
41. Edvinsson L, Krause DN. Cerebral Blood Flow and Metabolism. Philadelphia: Lippincott, Williams & Wilkins, 2002.
42. Esposito AG, Baker-Ward L, Mueller S. Interference Suppression vs. Response Inhibition: An Explanation for the Absence of a Bilingual Advantage in Preschoolers' Stroop Task Performance. Cogn Dev. 2013 Oct;28(4):354-363.
43. Fadel PJ, Ogoh S, Watenpaugh DE, Wasmund W, Olivencia-Yurvati A, Smith ML, Raven PB. Carotid baroreflex regulation of sympathetic nerve activity during dynamic exercise in humans. Am J Physiol Heart Circ Physiol. 2001 Mar;280(3):H1383-90.
44. Fadel PJ, Raven PB. Human investigations into the arterial and cardiopulmonary baroreflexes during exercise. Exp Physiol. 2012 Jan;97(1):39-50.
45. Flück D, Beaudin AE, Steinback CD, Kumarpillai G, Shobha N, McCreary CR, Peca S, Smith EE, Poulin MJ. Effects of aging on the association between cerebrovascular responses to visual stimulation, hypercapnia and arterial stiffness. Front Physiol. 2014 Feb 19;5:49.
46. Gatouillat A, Bleton H, VanSwearingen J, Perera S, Thompson S, Smith T, Sejdić E. Cognitive tasks during walking affect cerebral blood flow signal features in middle cerebral arteries and their correlation to gait characteristics. Behav Brain Funct. 2015 Sep 26;11(1):29.
47. Gledhill N, Cox D, Jamnik R. Endurance athletes' stroke volume does not plateau: major advantage is diastolic function. Med Sci Sports Exerc. 1994 Sep;26(9):1116-21.
48. González-Alonso J, Dalsgaard MK, Osada T, Volianitis S, Dawson EA, Yoshiga CC, Secher NH. Brain and central haemodynamics and oxygenation during maximal exercise in humans. J Physiol, 2004, 15; 557: 331-342.

49. Green AL, Paterson DJ. Identification of neurocircuitry controlling cardiovascular function in humans using functional neurosurgery: implications for exercise control. Exp Physiol. 2008 Sep;93(9):1022-8.
50. Guicciardi M, Crisafulli A, Doneddu A, Fadda D, Lecis R. Effects of Metabolic Syndrome on Cognitive Performance of Adults During Exercise. Front Psychol. 2019 Aug 8;10:1845.
51. Gupta AK, Menon DK, Czosnyka M, Smielewski P, Jones JG. Thresholds for hypoxic cerebral vasodilation in volunteers. Anesth Analg , 1997, 85: 817–820.
52. Harper AM, Deshmukh VD, Rowan JO, Jennett WB. The influence of sympathetic nervous activity on cerebral blood flow. Arch Neurol, 1972, 27:1–6.
53. Hartman YAW, Tillmans LCM, Benschop DL, Hermans ANL, Nijssen KMR, Eijsvogels TMH, Willems PHGM, Tack CJ, Hopman MTE, Claassen JAHR, Thijssen DHJ. Long-Term and Acute Benefits of Reduced Sitting on Vascular Flow and Function. Med Sci Sports Exerc. 2020 Aug 21,
54. Ichinose M, Maeda S, Kondo N, Nishiyasu T. Blood pressure regulation II: What happens when one system must serve two masters - Oxygen delivery and pressure regulation? European Journal of Applied Physiology. 2014 Mar;114(3):451-465.
55. Iellamo F. Baroreflex control of heart rate during exercise: a topic of perennial conflict. Journal of Applied Physiology. 2001, vol.90, no. 3, pp. 1184–1185.
56. Ishii K, Liang N, Asahara R, Takahashi M, Matsukawa K. Feedforward- and motor effort-dependent increase in prefrontal oxygenation during voluntary one-armed cranking. J Physiol. 2018 Nov;596(21):5099-5118. doi: 10.1113/JP276956. Epub 2018 Sep 30.
57. Istenes I, Körei AE, Putz Z, Németh N, Martos T, Keresztes K, Kempler MS, Erzsébet VO, Vargha P, Kempler P. Heart rate variability is severely impaired among type 2 diabetic patients with hypertension. Diabetes Metab Res Rev , 2014, 30(4): 305-312.
58. Jordan J, Shannon JR, Diedrich A, Black B, Costa F, Robertson D, Biaggioni I. Interaction of carbon dioxide and sympathetic nervous system activity in the regulation of cerebral perfusion in humans. Hypertension , 2000, 36: 383–388.
59. Joyner MJ. Preclinical and clinical evaluation of autonomic function in humans. J Physiol. 2016 Jul 15;594(14):4009-13. doi: 10.1113/JP271875. Epub 2016 May 27.
60. Kaufman MP, Longhurst JC, Rybicki KJ, Wallach JH, Mitchell JH. Effects of static muscular contraction on impulse activity of groups III and IV afferents in cats. J Appl Physiol Respir Environ Exerc Physiol. 1983 Jul;55(1 Pt 1):105-12.
61. Kim YS, Seifert T, Brassard P, Rasmussen P, Vaag A, Nielsen HB, Secher NH, van Lieshout JJ. Impaired cerebral blood flow and oxygenation during exercise in type 2 diabetic patients. Physiol Rep, 2015, 3(6).
62. Leenders KL, Perani D, Lammertsma AA, Heather JD, Buckingham P, Healy MJ, Gibbs JM,Wise RJ, Hatazawa J, Herold S, et al. Cerebral blood flow, blood volume and oxygen utilization. Normal values and effect of age. Brain, 1990 113, 27–47.
63. Levine BD, Zhang R. Sympathetic activity does/does not influence cerebral blood flow. Autonomic control of the cerebral circulation is most important for dynamic cerebral autoregulation. J Appl Physiol, 2008, 105:1369–1373.
64. Lewis SF, Taylor WF, Graham RM, Pettinger WA, Schutte JE, Blomqvist CG. Cardiovascular responses to exercise as functions of absolute and relative work load. J Appl Physiol Respir Environ Exerc Physiol. 1983 May;54(5):1314-23.

65. Liu-Ambrose T, Nagamatsu LS, Voss MW, Khan KM, Handy TC. Resistance training and functional plasticity of the aging brain: a 12-month randomized controlled trial. Neurobiol Aging. 2012 Aug;33(8):1690-8.
66. Lucas SJ, Tzeng YC, Galvin SD, Thomas KN, Ogoh S, Ainslie PN. Influence of changes in blood pressure on cerebral perfusion and oxygenation. Hypertension. 2010 Mar;55(3):698-705.
67. MacLeod DB, Ikeda K, Vacchiano C, Lobbestael A, Wahr JA, Shaw AD. Development and validation of a cerebral oximeter capable of absolute accuracy. J Cardiothorac Vasc Anesth. 2012 Dec;26(6):1007-14.
68. Manzella D, Grella R, Abbatecola AM, Paolisso G. Repaglinide administration improves brachial reactivity in type 2 diabetic patients. Diabetes Care. 2005 Feb;28(2):366-71.
69. McDonald DA. Blood Flow in Arteries. London: Arnold, 1974, p. 450.
70. Michael S, Graham KS, Davis GM Oam. Cardiac Autonomic Responses during Exercise and Post-exercise Recovery Using Heart Rate Variability and Systolic Time Intervals-A Review. Front Physiol. 2017 May 29;8:301.
71. Milia R, Roberto S, Mulliri G, Loi A, Marcelli M, Sainas G, Milia N, Marongiu E, Crisafulli A. Effect of aging on hemodynamic response to metaboreflex activation. Eur J Appl Physiol. 2015 Aug;115(8):1693-703.
72. Milia R, Velluzzi F, Roberto S, Palazzolo G, Sanna I, Sainas G, Pusceddu M, Mulliri G, Loviselli A, Crisafulli A. Differences in hemodynamic response to metaboreflex activation between obese patients with metabolic syndrome and healthy subjects with obese phenotype. Am J Physiol Heart Circ Physiol. 2015 Sep;309(5):H779-89.
73. Murphy MN, Mizuno M, Mitchell JH, Smith SA. Cardiovascular regulation by skeletal muscle reflexes in health and disease. Am J Physiol Heart Circ Physiol, 2011, 301: H1191–H1204.
74. Nasreddine ZS, Phillips NA, Bédirian V, Charbonneau S, Whitehead V, Collin I, Cummings JL, Chertkow H. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. J Am Geriatr Soc. 2005 Apr;53(4):695-9.
75. Nishiyasu T, Tan N, Morimoto K, Nishiyasu M, Yamaguchi Y, Murakami N. Enhancement of parasympathetic cardiac activity during activation of muscle metaboreflex in humans. J Appl Physiol (1985). 1994 Dec;77(6):2778-83.
76. Nobrega AC, O'Leary D, Silva BM, Marongiu E, Piepoli MF, Crisafulli A. Neural regulation of cardiovascular response to exercise: role of central command and peripheral afferents. Biomed Res Int. 2014;2014:478965.
77. Ogoh S, Fisher JP, Dawson EA, White MJ, Secher NH, Raven PB. Autonomic nervous system influence on arterial baroreflex control of heart rate during exercise in humans. J Physiol. 2005 Jul 15;566(Pt 2):599-611.
78. Ogoh S, Sato K, Hirasawa A, Sadamoto T. The effect of muscle metaboreflex on the distribution of blood flow in cerebral arteries during isometric exercise. J Physiol Sci. 2019 Mar;69(2):375-385.
79. O'Leary DS. Autonomic mechanisms of muscle metaboreflex control of heart rate. J Appl Physiol (1985). 1993 Apr;74(4):1748-54.
80. Olufsen MS, Nadim A, Lipsitz LA. Dynamics of cerebral blood flow regulation explained using a lumped parameter model. Am J Physiol Regul Integr Comp Physiol, 2002,282: R611–R622.

81. Paulson OB, Strandgaard S, Edvinsson L. Cerebral autoregulation. Cerebrovasc Brain Metab Rev, 1990, 2: 161–192.
82. Piepoli M, Clark AL, Coats AJS. Muscle metaboreceptors in hemodynamic, autonomic, and ventilatory responses to exercise in men. Am J Physiol 1995; 269: H1428-36.
83. Piepoli MF, Crisafulli A. Pathophysiology of human heart failure: importance of skeletal muscle myopathy and reflexes. Exp Physiol. 2014 Apr;99(4):609-15.
84. Plichta MM, Herrmann MJ, Ehlis AC, Baehne CG, Richter MM, Fallgatter AJ. Event-related visual versus blocked motor task: detection of specific cortical activation patterns with functional near-infrared spectroscopy. Neuropsychobiology. 2006;53(2):77-82.
85. Poitras VJ, Hudson RW, Tschakovsky ME. Exercise intolerance in Type 2 diabetes: is there a cardiovascular contribution? J Appl Physiol (1985) 2018; 124(5): 1117-1139.
86. Rasmussen P, Nielsen J, Overgaard M, Krogh-Madsen R, Gjedde A, Secher NH, Petersen NC. Reduced muscle activation during exercise related to brain oxygenation and metabolism in humans. J Physiol. 2010, 588, 1985–1995.
87. Roberto S, Crisafulli A. Consequences of Type 1 and 2 Diabetes Mellitus on the Cardiovascular Regulation During Exercise: A Brief Review. Curr Diabetes Rev. 2017 Oct 11;13(6):560-565.
88. Roberto S, Marongiu E, Pinna M, Angius L, Olla S, Bassareo P, Tocco F, Concu A, Milia R, Crisafulli A. Altered hemodynamics during muscle metaboreflex in young type 1 diabetes patients. J Appl Physiol (1985). 2012 Oct 15;113(8):1323-31.
89. Roberto S, Milia R, Doneddu A, Pinna V, Palazzolo G, Serra S, Orrù A, Hosseini Kakhak SA, Ghiani G, Mulliri G, Pagliaro P. Crisafulli A. Hemodynamic Abnormalities During Muscle Metaboreflex Activation in Type 2 Diabetes Mellitus Patients. J Appl Physiol. 2019, 126(2): 444-453.
90. Rowell LB, O'Leary DS. Reflex control of the circulation during exercise: chemoreflexes and mechanoreflexes. J Appl Physiol (1985). 1990 Aug;69(2):407-18.
91. Rozanski A, Bairey CN, Krantz DS, Friedman J, Resser KJ, Morell M, Hilton-Chalfen S, Hestrin L, Bietendorf J, Berman DS. Mental stress and the induction of silent myocardial ischemia in patients with coronary artery disease. N Engl J Med. 1988; 318(16): 1005-1012.
92. Sagawa K, Eisner A. Static pressure flow relation in the total systemic vascular bed of the dog and its modification by the baroreceptor reflex. Circulation Research, 1975, vol. 36, no. 3, pp.406–413.
93. Sainas G, Milia R, Palazzolo G, Ibba G, Marongiu E, Roberto S, Pinna V, Ghiani G, Tocco F, Crisafulli A. Mean Blood Pressure Assessment during Post-Exercise: Result from Two Different Methods of Calculation. J Sports Sci Med. 2016 Aug 5;15(3):424-433.
94. Sanna I, Pinna V, Milia R, Roberto S, Olla S, Mulliri G, Crisafulli A. Hemodynamic Responses during Enduro-Motorcycling Performance. Front Physiol. 2017 Dec 14;8:1062.
95. Scheel P, Ruge C, Petruch UR, Schoning M. Color duplex measurement of cerebral blood flow volume in healthy adults. Stroke, 2000, 31, 147–150.
96. Schillaci G, Pirro M, Pucci G, Mannarino MR, Gemelli F, Siepi D, Vaudo G, Mannarino E. Different impact of the metabolic syndrome on left ventricular structure and function in hypertensive men and women. Hypertension. 2006 May;47(5):881-6.
97. Secher NH, Seifert T, Van Lieshout JJ. Cerebral blood flow and metabolism during exercise: implications for fatigue. J Appl Physiol (1985), 2008, 104(1): 306-314.
98. Selvin E, Parinello CM. Age-related differences in glycaemic control in diabetes. Diabetologia. 2013, Dec;56(12):2549-51.

99. Simon SS, Hampstead BM, Nucci MP, Duran FLS, Fonseca LM, Martin MDGM, Ávila R, Porto FHG, Brucki SMD, Martins CB, Tascone LS, Amaro E Jr, Busatto GF, Bottino CMC. Cognitive and Brain Activity Changes After Mnemonic Strategy Training in Amnestic Mild Cognitive Impairment: Evidence From a Randomized Controlled Trial. Front Aging Neurosci. 2018 Nov 13;10:342.
100. Skirton H, Chamberlain W, Lawson C, Ryan H, Young E. A systematic review of variability and reliability of manual and automated blood pressure readings. J Clin Nurs. 2011 Mar;20(5-6):602-14.
101. Smith KJ, Ainslie PN. Regulation of cerebral blood flow and metabolism during exercise. Exp Physiol. 2017 Nov 1;102(11):1356-1371.
102. Strangman G, Boas DA, Sutton JP. Non-invasive neuroimaging using near-infrared light. Biol Psychiatry. 2002 Oct 1;52(7):679-93.
103. Strangman G, Culver JP, Thompson JH, Boas DA. A quantitative comparison of simultaneous BOLD fMRI and NIRS recordings during functional brain activation. Neuroimage. 2002 Oct;17(2):719-31.
104. Suzuki M, Miyai I, Ono T, et al. Prefrontal and premotor cortices are involved in adapting walking and running speed on the treadmill: an optical imaging study. Neuroimage. 2004 Nov;23(3):1020-1026.
105. Tarumi T, Zhang R. Cerebral blood flow in normal aging adults: cardiovascular determinants, clinical implications, and aerobic fitness. J Neurochem. 2018 Mar;144(5):595-608.
106. Thomas SN, Schroeder T, Secher NH, Mitchell JH. Cerebral blood flow during submaximal and maximal dynamic exercise in humans. J Appl Physiol (1985). 1989 Aug;67(2):744-8.
107. Van Lieshout JJ, Secher NH. Sympathetic activity does/does not influence cerebral blood flow. J Appl Physiol , 2008, 105: 1364–1366.
108. Vvan Mil AC, Hartman Y, van Oorschot F, Heemels A, Bax N, Dawson EA, Hopkins N, Hopman MT, Green DJ, Oxborough DL, Thijssen DH. Correlation of carotid artery reactivity with cardiovascular risk factors and coronary artery vasodilator responses in asymptomatic, healthy volunteers. J Hypertens. 2017 May;35(5):1026-1034.
109. Verner M, Herrmann MJ, Troche SJ, Roebers CM, Rammsayer TH. Cortical oxygen consumption in mental arithmetic as a function of task difficulty: a near-infrared spectroscopy approach. Front Hum Neurosci. 2013 May 22;7:217.
110. Vianna LC, Deo SH, Jensen AK, Holwerda SW, Zimmerman MC, Fadel PJ. Impaired dynamic cerebral auto-regulation at rest and during isometric exercise in type 2 diabetes patients. Am J Physiol (Heart Circ Physiol), 2015, 308: H681-H687.
111. Vianna LC, Fisher JP. Reflex control of the cardiovascular system during exercise in disease. Curr Opinion Physiol, 2019; 10: 110-117.
112. Waldron M, David Patterson S, Jeffries O. Inter-Day Reliability of Finapres. Sports Med Int Open. 2018;2(1):E9-E15.
113. Wasmund WL, Westerholm EC, Watenpaugh DE, Wasmund SL, Smith ML. Interactive effects of mental and physical stress on cardiovascular control. J Appl Physiol (1985). 2002 May;92(5):1828-34.
114. Webb HE, Rosalky DA, McAllister MJ, Acevedo EO, Kamimori GH. Aerobic fitness impacts sympathoadrenal axis responses to concurrent challenges. Eur J Appl Physiol. 2017 Feb;117(2):301-313.

115. Wei EP, Kontos HA, Patterson JL Jr. Dependence of pial arteriolar response to hypercapnia on vessel size. Am J Physiol Heart Circ Physiol , 1980, 238: H697–H703.
116. White DW, Raven PB. Autonomic neural control of heart rate during dynamic exercise: revisited. J Physiol. 2014 Jun 15;592(12):2491-500.
117. Willie CK, Tzeng YC, Fisher JA, Ainslie PN. Integrative regulation of human brain blood flow. J Physiol. 2014 Mar 1;592(5):841-59.
118. Willie CK. "Utility of transcranial Doppler ultrasound for the integrative assessment of cerebrovascular function." J Neurosci Methods, 2011, 196(2): 221-237.
119. Xiong XQ, Chen WW, Zhu GO. Adipose afferent reflex: sympathetic activation, and obesity hypertension. Acta Physiol. 2014; 210: 468–478.
120. Yaffe K, Kanaya A, Lindquist K, Simonsick EM, Harris T, Shorr RI, Tylavsky FA, Newman AB. The metabolic syndrome, inflammation, and risk of cognitive decline. JAMA. 2004 Nov 10;292(18):2237-42.
121. Yew B, DA Nation. "Cerebrovascular resistance: effects on cognitive decline, cortical atrophy, and progression to dementia." Brain, 2017, 140(7): 1987-2001.
122. Zhang R, Zuckerman JH, Iwasaki K, Wilson TE, Crandall CG, Levine BD. Autonomic neural control of dynamic cerebral autoregulation in humans. Circulation, 2002, 106: 1814–1820.
123. Zonneveld HI, Loehrer EA, Hofman A, Niessen WJ, van der Lugt A, Krestin GP, Ikram MA, Vernooij MW. The bidirectional association between reduced cerebral blood flow and brain atrophy in the general population. J Cereb Blood Flow Metab. 2015 Nov;35(11):1882-7.

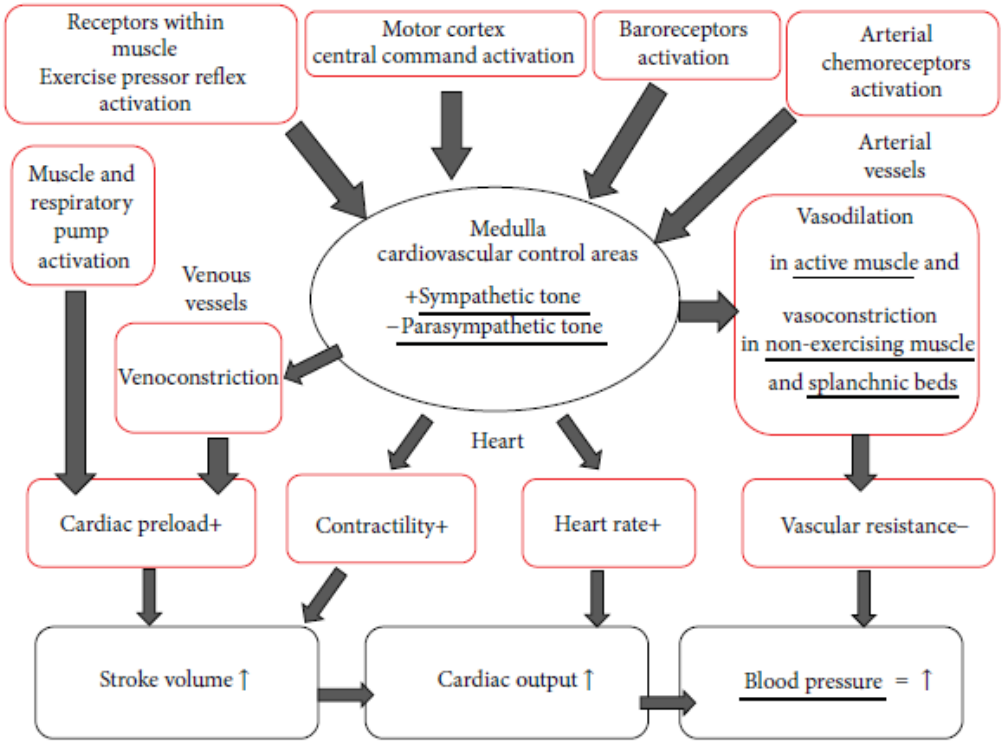
Introduction – Figures Captions

Figure I. A Cardiovascular adjustment during dynamic exercise. Impulses from motor cortex, contracting muscle, baroreceptors, and chemoreceptors converge on the cardiovascular control areas situated in the medulla (Nobrega et al.2014). **B** Interactions between the three main neural reflexes operating during exercise.

Figure II. Schematic representation of the aims of the research.

Figure I

A


Receptors within muscle
Exercise pressor reflex activation
Motor cortex
central command activation
Baroreceptors activation
Arterial chemoreceptors activation
Arterial vessels
Muscle and respiratory pump activation
Medulla
cardiovascular control areas
+Sympathetic tone
−Parasympathetic tone
Vasodilation
in active muscle and
vasoconstriction
in non-exercising muscle
and splanchnic beds
Venous vessels
Venoconstriction
Heart
Cardiac preload+
Contractility+
Heart rate+
Vascular resistance−
Stroke volume ↑
Cardiac output ↑
Blood pressure = ↑

B

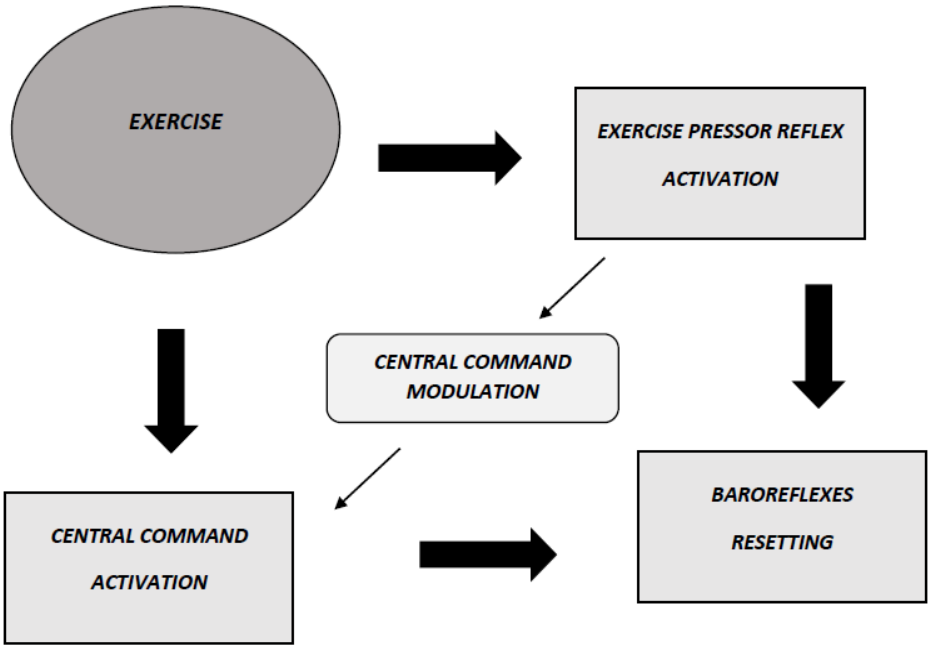
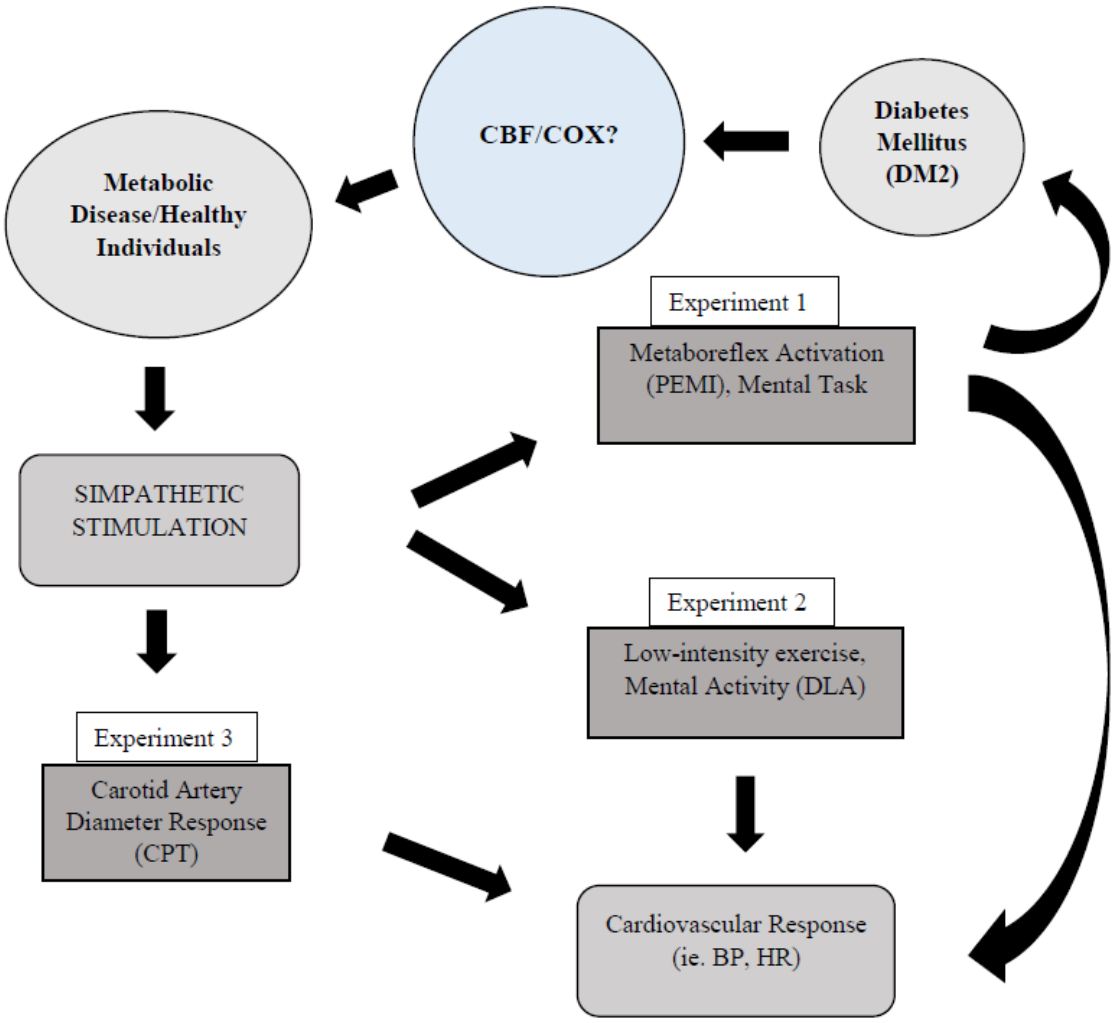

EXERCISE
EXERCISE PRESSOR REFLEX ACTIVATION
CENTRAL COMMAND MODULATION
CENTRAL COMMAND ACTIVATION
BAROREFLEXES RESETTING

Figure II


CBF/COX?
Diabetes Mellitus (DM2)
Metabolic Disease/Healthy Individuals
Experiment 1
Metaboreflex Activation (PEMI), Mental Task
SIMPATHETIC STIMULATION
Experiment 2
Low-intensity exercise, Mental Activity (DLA)
Experiment 3
Carotid Artery Diameter Response (CPT)
Cardiovascular Response (ie. BP, HR)

Experiment 1 - Figure captions

Figure 1. Schematic representation of the various sessions in the study protocol. PEMI: post-exercise muscle ischemia; CER: control exercise recovery; MT: mental task; CER+MT: control exercise recovery + mental task; PEMI+MT: post-exercise muscle ischemia + mental task.

Figure 2. Hemodynamic data during control exercise recovery (CER), post-exercise muscle ischemia (PEMI), mental task (MT), control exercise recovery + mental task (CER+MT), and post-exercise muscle ischemia + mental task (PEMI+MT) tests in the type 2 Diabetes Mellitus (DM2, n= 13) and the control (CTL, n= 13) groups. Data were gathered at the 9th minute of the sessions. HR= heart rate (panel A); SV= stroke volume (panel B); CO= cardiac output (panel C). Values are mean ± SD. A horizontal bracket indicates the overall main effect of the groups.

Figure 3. Hemodynamic data during control exercise recovery (CER), post-exercise muscle ischemia (PEMI), mental task (MT), control exercise recovery + mental task (CER+MT), and post-exercise muscle ischemia + mental task (PEMI+MT) tests in the type 2 Diabetes Mellitus (DM2, n= 13) and the control (CTL, n= 13) groups. Data were gathered at the 9th minute of the sessions. VFR= ventricular filling rate (panel A); VER= ventricular emptying rate (panel B); MAP= mean arterial pressure (panel C). Values are mean ± SD. A horizontal bracket indicates the overall main effect of the groups; a vertical bracket indicates the overall main effect of the condition. *= $p<0.05$ vs. CER test of the DM2 group.

Figure 4. Hemodynamic data during control exercise recovery (CER), post-exercise muscle ischemia (PEMI), mental task (MT), control exercise recovery + mental task (CER+MT), and post-exercise muscle ischemia + mental task (PEMI+MT) tests in the type 2 Diabetes Mellitus (DM2, n= 13) and the control (CTL, n= 13) groups. Data were gathered at the 9th minute of the sessions. SVR= systemic vascular resistance (panel A); PEP= pre-ejection period (panel B); COX= cerebral oxygenation (panel C). In panel C, a dotted line indicates rest level. Values are mean ± SD. The horizontal brackets indicate the overall main effect of the groups; a vertical bracket indicates the overall main effect of the condition. *= $p<0.05$ vs. CER test of the same group; †= $p<0.05$ vs. CER, PEMI, and MT tests of the same group; ‡ $p<0.05$ vs. CER, PEMI, MT, CER+MT, and PEMI+MT tests of the DM2 group.

Figure 1

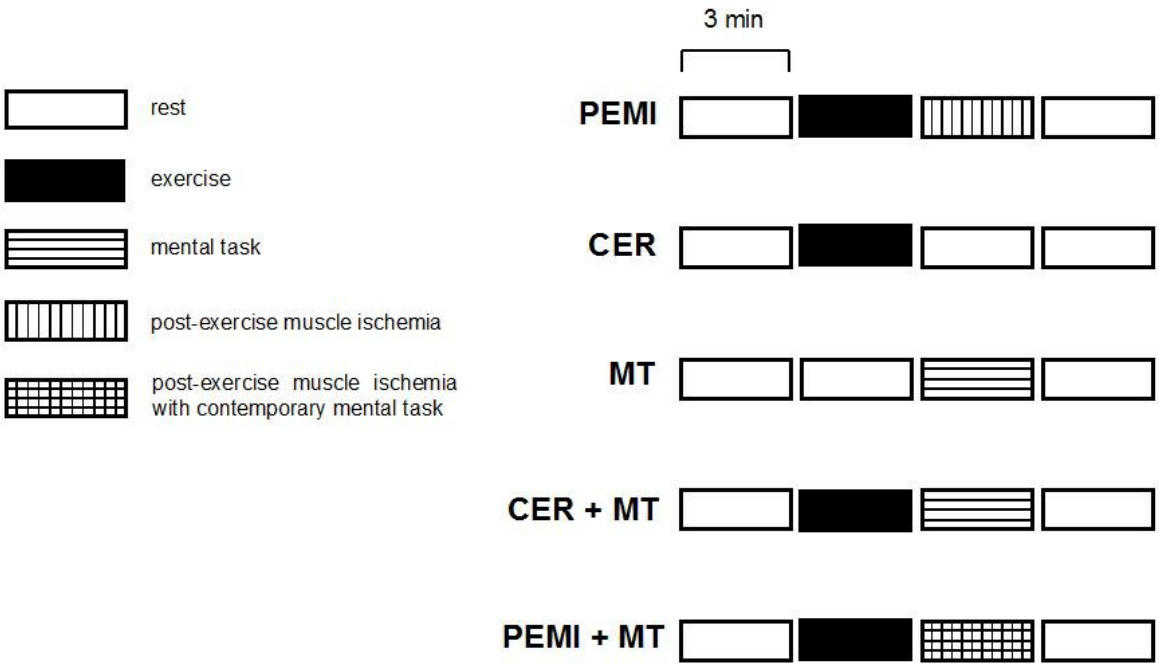

3 min
rest
exercise
mental task
post-exercise muscle ischemia
post-exercise muscle ischemia with contemporary mental task
PEMI
CER
MT
CER + MT
PEMI + MT

Figure 2

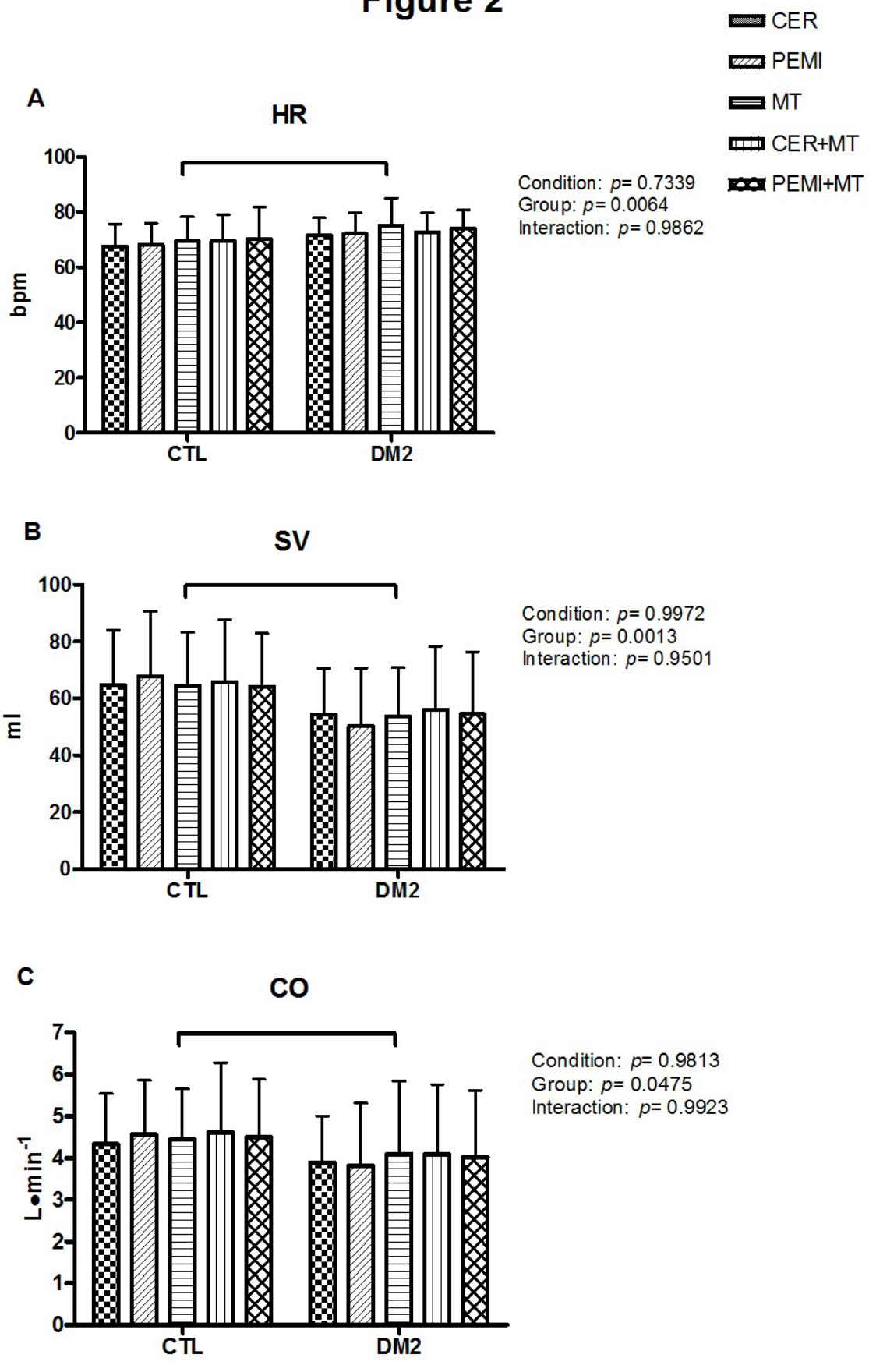

CER
PEMI
MT
CER+MT
PEMI+MT
A
HR
bpm
100
80
60
40
20
0
CTL
DM2
Condition: p= 0.7339
Group: p= 0.0064
Interaction: p= 0.9862
B
SV
ml
100
80
60
40
20
0
CTL
DM2
Condition: p= 0.9972
Group: p= 0.0013
Interaction: p= 0.9501
C
CO
L•min-1
7
6
5
4
3
2
1
0
CTL
DM2
Condition: p= 0.9813
Group: p= 0.0475
Interaction: p= 0.9923

Figure 3

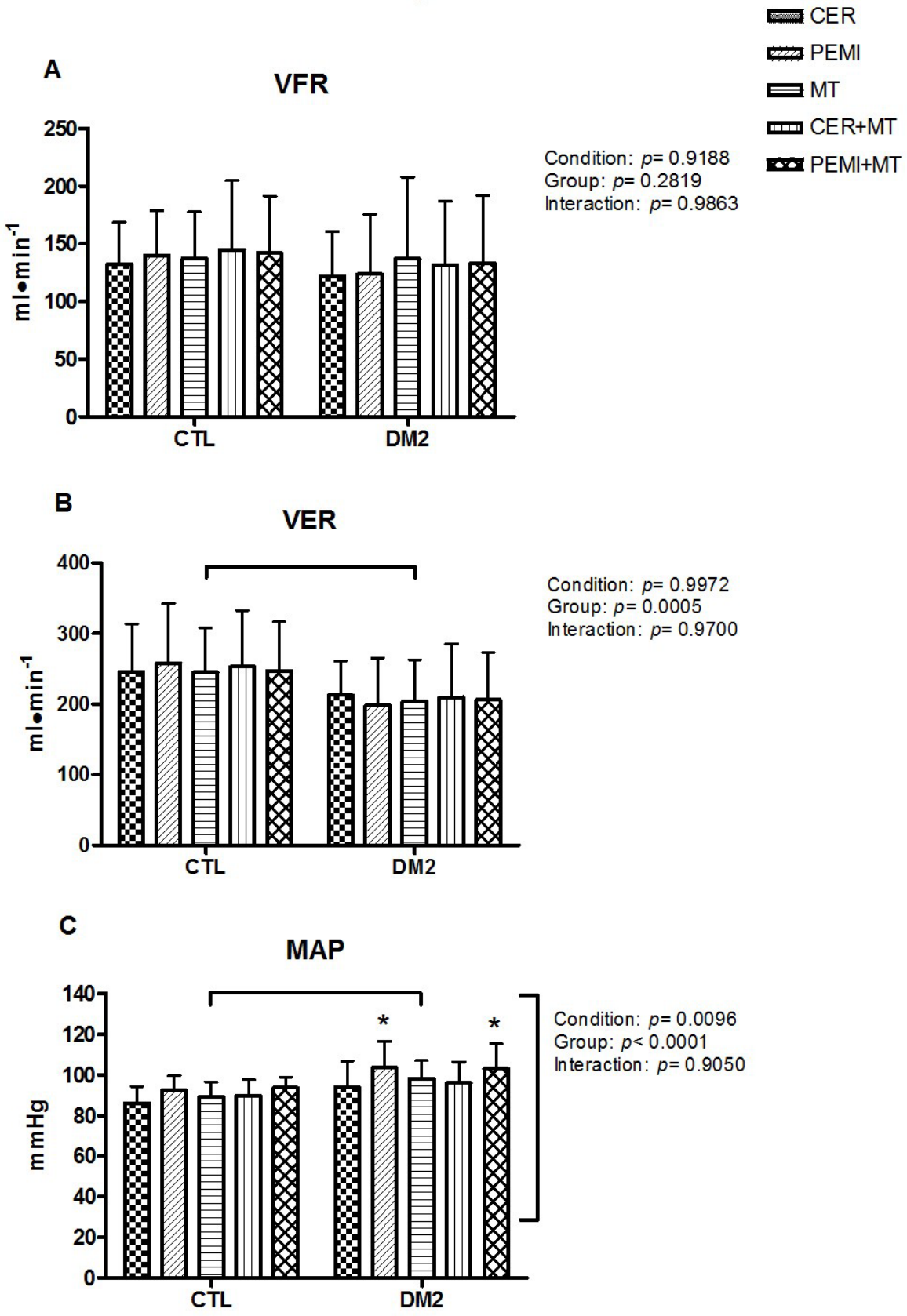

CER
PEMI
MT
CER+MT
PEMI+MT
A
VFR
ml•min-1
250
200
150
100
50
0
CTL
DM2
Condition: p= 0.9188
Group: p= 0.2819
Interaction: p= 0.9863
B
VER
ml•min-1
400
300
200
100
0
CTL
DM2
Condition: p= 0.9972
Group: p= 0.0005
Interaction: p= 0.9700
C
MAP
mmHg
140
120
100
80
60
40
20
0
*
*
CTL
DM2
Condition: p= 0.0096
Group: p< 0.0001
Interaction: p= 0.9050

Figure 4

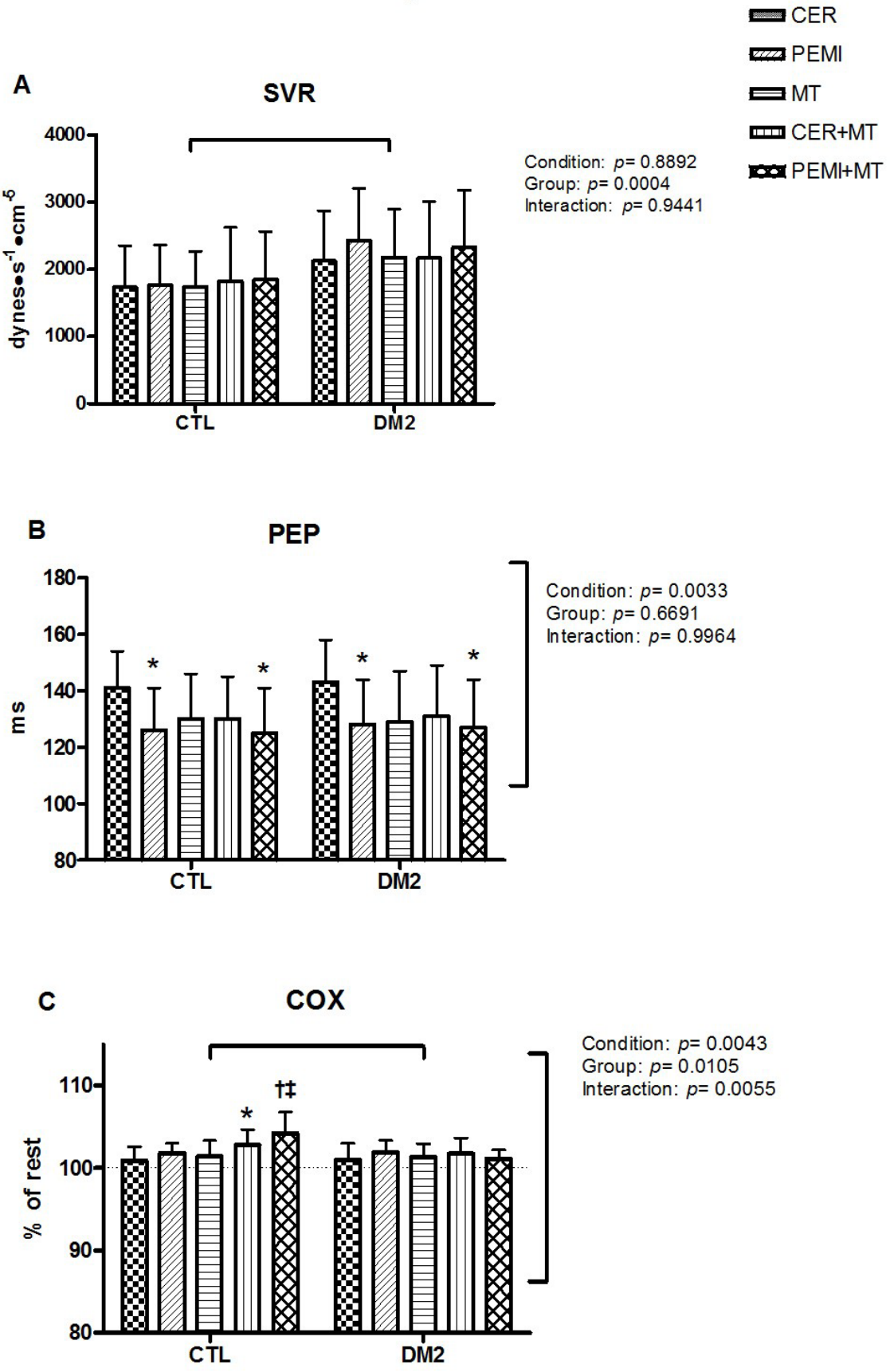

CER
PEMI
MT
CER+MT
PEMI+MT
A
SVR
dynes•s-1•cm-5
4000
3000
2000
1000
0
CTL
DM2
Condition: p= 0.8892
Group: p= 0.0004
Interaction: p= 0.9441
B
PEP
ms
180
160
140
120
100
80
*
CTL
DM2
Condition: p= 0.0033
Group: p= 0.6691
Interaction: p= 0.9964
C
COX
% of rest
110
100
90
80
*
†‡
CTL
DM2
Condition: p= 0.0043
Group: p= 0.0105
Interaction: p= 0.0055

Table 1. Anthropometric characteristics of groups together with results of the preliminary medical examination and of cardiopulmonary test. CTL= controls (n= 13), DM2= type 2 Diabetes Mellitus (n= 13). Values are mean ± SD.

	CTL	DM2	*p* value
Height (cm)	170.6±9.6	171.0±10.3	0.9193
Body mass (kg)	69.2±12.4	90.4±19.9	0.0033
Body mass index (kg/m^2)	23.5±2.5	30.9±5.69	0.0002
Systolic blood pressure (mmHg)	112.8±11.2	124.6±15.5	0.0358
Diastolic blood pressure (mmHg)	76.8±6.9	80.2±11.5	0.3088
Fasting glucose (mg/dL)	85.2±8.8	108.0±13.4	<0.001
Maximal O_2 uptake (mL/kg/min)	34.2±7.6	17.0±6.2	<0.001
Maximum workload (W)	210.5±75.8	120.0±49.6	0.0015
Maximum heart rate (bpm)	158.6±15.6	139.2±19.0	0.0089

Table 2. Hemodynamic and cerebral oxygenation (COX) data during rest periods preceding post-exercise control exercise recovery (CER), muscle ischemia (PEMI), mental task (MT), control exercise recovery + mental task (CER+MT), and post-exercise muscle ischemia + mental task (PEMI+MT) tests for control (CTL, n= 13) and type 2 Diabetes Mellitus (DM2, n=13) groups. HR= heart rate; SV= stroke volume; CO= cardiac output; VFR= ventricular filling rate; VER= ventricular emptying rate; SVR= systemic vascular resistance; MAP= mean arterial pressure; PEP= pre-ejection period. Values are mean ± SD.

	CER	PEMI	MT	CER+MT	PEMI+MT	*p* value condition effect	*p* value group effect	*p* value interaction
HR (bpm)	CTL 67.04 ±7.90 DM2 74.61±9.51	CTL 68.04 ±7.84 DM2 73.55±7.85	CTL 69.22±11.64 DM2 72.54±7.34	CTL 67.36±8.82 DM2 74.52±9.48	CTL 68.74±10.25 DM2 72.91±7.60	1.000	< 0.001	0.893
SV (ml)	CTL 60.56±16.46 DM2 56.57±19.34	CTL 60.74±16.46 DM2 55.39±21.44	CTL 65.54±21.67 DM2 58.47±22.69	CTL 64.76±21.72 DM2 54.97±18.04	CTL 65.93±20.75 DM2 55.57±23.04	0.956	0.042	0.975
CO ($L \cdot min^{-1}$)	CTL 4.03±1.05 DM2 4.22±1.56	CTL 4.08±1.05 DM2 4.03±1.55	CTL 4.48±1.47 DM2 4.23±1.65	CTL 4.35±1.44 DM2 4.13±1.69	CTL 4.51±1.45 DM2 4.00±1.63	0.955	0.518	0.937
VFR ($ml \cdot s^{-1}$)	CTL 121.62±34.27 DM2 139.32±58.05	CTL 122.83±33.40 DM2 130.42±51.13	CTL 138.55±52.35 DM2 137.57±57.69	CTL 130.75±45.91 DM2 136.49±61.35	CTL 139.11±49.28 DM2 129.86±57.37	0.944	0.642	0.907
VER ($ml \cdot s^{-1}$)	CTL 235.32±57.94 DM2 218.45±62.87	CTL 242.17±68.78 DM2 215.51±71.28	CTL 262.73±87.58 DM2 220.59±73.21	CTL 262.08±76.83 DM2 211.95±78.37	CTL 261.39±75.22 DM2 215.64±78.37	0.935	0.004	0.911
SVR ($dynes \cdot s^{-1} \cdot cm^{-5}$)	CTL 1803.56±579.53 DM2 1981.60±712.97	CTL 1781.88±581.85 DM2 2104.44±669.74	CTL 1654.63±554.12 DM2 2033.64±844.17	CTL 1753.24±740.67 DM2 2021.22±676.83	CTL 1687.80±616.56 DM2 2087.83±814.16	0.991	0.011	0.978
MAP (mmHg)	CTL 84.41±8.93 DM2 93.02±12.49	CTL 84.40±8.05 DM2 94.76±10.98	CTL 83.15±8.08 DM2 92.85±15.49	CTL 83.82±7.96 DM2 92.97±12.03	CTL 85.90±8.33 DM2 91.43±14.34	0.990	<0.001	0.943
PEP (ms)	CTL 0.138±0.01 DM2 0.131±0.03	CTL 0.136±0.01 DM2 0.138±0.03	CTL 0.142±0.02 DM2 0.133±0.03	CTL 0.140±0.01 DM2 0.134±0.04	CTL 0.140±0.02 DM2 0.134±0.03	0.993	0.282	0.970
COX	CTL 69.71±5.68 DM2 67.50±5.79	CTL 69.92±5.45 DM2 67.40±5.89	CTL 69.90±5.89 DM2 66.90±5.57	CTL 69.66±5.24 DM2 67.40±5.93	CTL 69.92±5.81 DM2 67.52±5.82	0.999	0.014	0.999

Table 3. Hemodynamic and cerebral oxygenation (COX, expressed as % changes with respect to rest) data values during the third minute of exercise (dynamic handgrip) of control exercise recovery (CER), muscle ischemia (PEMI), mental task (MT), control exercise recovery + mental task (CER+MT), and post-exercise muscle ischemia + mental task (PEMI+MT) tests for control (CTL, n= 13) and type 2 Diabetes Mellitus (DM2, n=13) groups. HR= heart rate; SV= stroke volume; CO= cardiac output; VFR= ventricular filling rate; VER= ventricular emptying rate; SVR= systemic vascular resistance; MAP= mean arterial pressure; PEP= pre-ejection period. Values are mean ± SD. *= $p < 0.05$ vs. MT test of the same group.

	CER	PEMI	MT	CER+MT	PEMI+MT	*p* value condition effect	*p* value group effect	*p* value interaction
HR (bpm)	CTL 71.83 ±8.21 DM2 79.73±8.92	CTL 71.36 ±7.38 DM2 80.88±12.03	CTL 69.11±10.43 DM2 72.89±7.31	CTL 73.42±9.42 DM2 80.31±11.52	CTL 73.10±11.93 DM2 80.53±9.77	0.1736	< 0.001	0.8803
SV (ml)	CTL 60.38±19.64 DM2 54.72±17.16	CTL 63.38±19.93 DM2 53.22±17.76	CTL 64.96±17.93 DM2 56.05±19.08	CTL 66.74±26.25 DM2 54.13±16.37	CTL 61.70±19.83 DM2 53.69±19.97	0.9647	0.0094	0.9780
CO ($L \cdot min^{-1}$)	CTL 4.26±1.17 DM2 4.36±1.52	CTL 4.46±1.15 DM2 4.34±1.72	CTL 4.45±1.27 DM2 4.08±1.55	CTL 4.85±1.77 DM2 4.35±1.61	CTL 4.48±1.42 DM2 4.30±1.64	0.9440	0.4210	0.9604
VFR ($ml \cdot s^{-1}$)	CTL 131.02±36.10 DM2 149.49±60.14	CTL 138.34±37.42 DM2 157.10±75.84	CTL 138.21±47.45 DM2 131.65±56.73	CTL 155.98±63.76 DM2 150.92±66.08	CTL 143.20±51.53 DM2 155.71±70.70	0.7869	0.4551	0.8754
VER ($ml \cdot s^{-1}$)	CTL 241.18±69.72 DM2 217.31±51.57	CTL 251.53±70.35 DM2 207.91±62.83	CTL 252.93±71.70 DM2 209.76±62.57	CTL 260.99±82.11 DM2 210.57±56.45	CTL 241.34±65.87 DM2 209.76±62.60	0.9878	0.0012	0.9541
SVR ($dynes \cdot s^{-1} \cdot cm^{-5}$)	CTL 1834.07±585.65 DM2 2200.05±732.41	CTL 1803.73±627.07 DM2 2370.47±1077.40	CTL 1661.30±544.59 DM2 2169.73±625.08	CTL1834.23±802.49 DM2 2231.05±671.34	CTL 1889.08±753.22 DM2 2176.59±715.04	0.9417	0.012	0.9608
MAP (mmHg)	CTL 93.72±9.99 * DM2 108.31±14.50 *	CTL 94.72±6.31 * DM2 109.59±14.25 *	CTL 83.30±6.67 DM2 95.91±13.31	CTL 94.54±8.06 * DM2 109.88±14.28 *	CTL 94.80±6.99 * DM2 106.85±14.21 *	0.0001	<0.001	0.9866
PEP (ms)	CTL 0.123±0.013 DM2 0.120±0.012 *	CTL 0.123±0.016 DM2 0.121±0.013	CTL 0.136±0.016 DM2 0.134±0.010	CTL 0.120±0.019 * DM2 0.123±0.013	CTL 0.121±0.015 * DM2 0.122±0.012	0.9066	0.0048	0.9359
COX (%)	CTL 102.69±2.52 * DM2 102.60±1.70 *	CTL 102.81±1.98 * DM2 102.86±2.02 *	CTL 100.79±1.41 DM2 100.36±1.10	CTL 102.91±2.46 * DM2 103.10±2.89 *	CTL 103.30±2.29 * DM2 102.24±2.00 *	0.0002	0.4674	0.8325

Experiment 2 - Figure captions

Figure 2.1 Schematic representation of the various sessions in the study protocol. SIT: sitting; STAND: standing; WALK15: walking at 15% of HRmax; WALK30: walking at 30% of HRmax

Figure 2.2 The effect of standing and walking and the age on the CBFV (cm/s), BP (mmHg) and C (cm/s/mmHg). In all 9 graphs the average is presented with the SEM. The black column represent the YG, while the white column represent the A group. Pcondition represents the effect of standing and walking when compared to sit, Pgroup represents the effect of age and Pgroup*condition represents the effect of standing and walking between both groups. Figure 1A: effect of STAND on CBFV, 1B: effect of WALK15 on CBFV, 1C: effect of WALK30 on CBFV, 1D: effect of STAND on BP, 1E: effect of WALK15 on BP, 1F: effect of WALK30 on BP, 1G: effect of STAND on conductance, 1H: effect of WALK15 on conductance, 1I: effect of WALK30 on conductance.

Figure 2.3. The effect of walking and the age on the CBFV (cm/s), BP (mmHg) and Conductance (cm/s/mmHg). In all 3 graphs the average is presented with the SEM. Pcondition represents the effect of walking when compared to standing, Pgroup represents the effect of age and Pgroup*condition represents the effect of walking between both groups. Figure 2A: effect in relative values on CBFV, 2B effect in relative values on BP, 2C effect in relative values on Conductance.

Figure 2.1

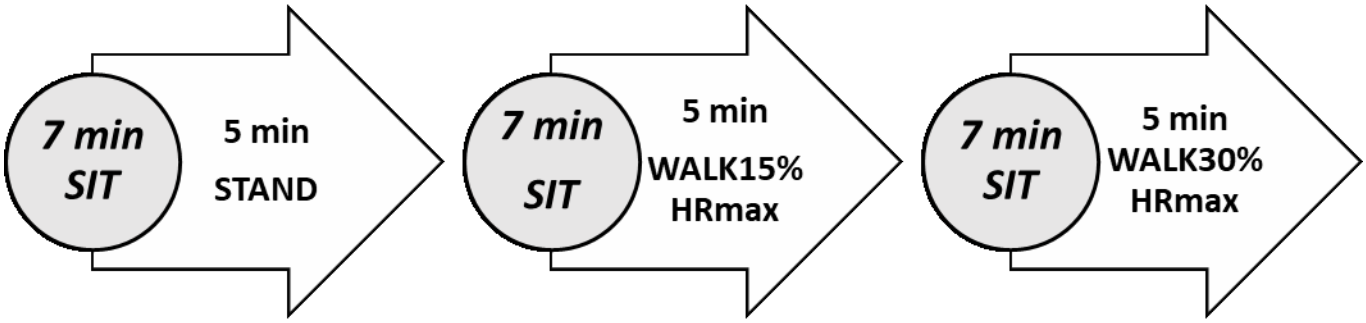

7 min SIT
5 min STAND
7 min SIT
5 min WALK15% HRmax
7 min SIT
5 min WALK30% HRmax

Figure 2.2

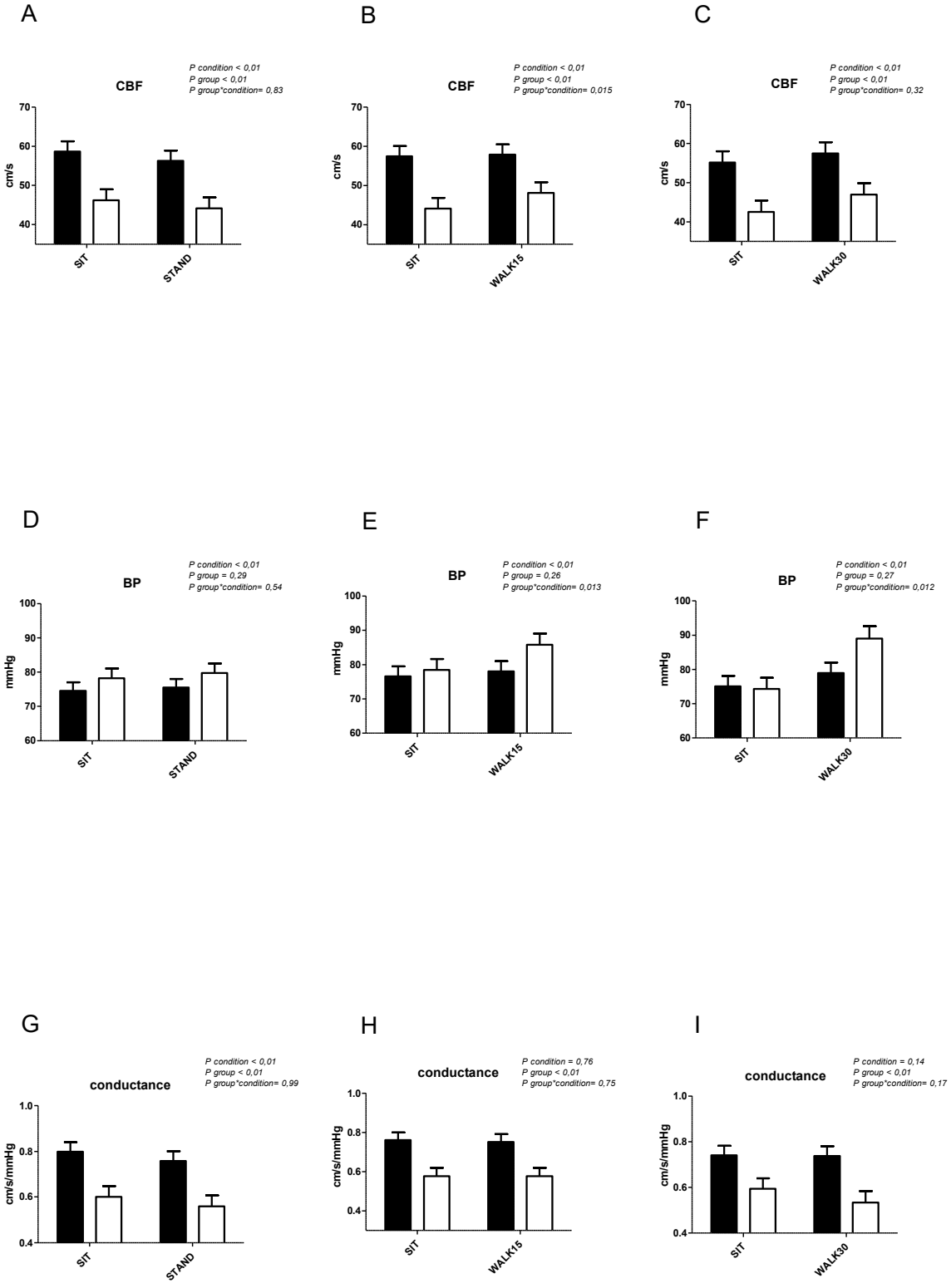

A
CBF
P condition < 0,01
P group < 0,01
P group*condition= 0,83
cm/s
70
60
50
40
SIT
STAND
B
CBF
P condition < 0,01
P group < 0,01
P group*condition= 0,015
cm/s
SIT
WALK15
C
CBF
P condition < 0,01
P group < 0,01
P group*condition= 0,32
cm/s
SIT
WALK30
D
BP
P condition < 0,01
P group = 0,29
P group*condition= 0,54
mmHg
100
90
80
70
60
SIT
STAND
E
BP
P condition < 0,01
P group = 0,26
P group*condition= 0,013
mmHg
SIT
WALK15
F
BP
P condition < 0,01
P group = 0,27
P group*condition= 0,012
mmHg
SIT
WALK30
G
conductance
P condition < 0,01
P group < 0,01
P group*condition= 0,99
cm/s/mmHg
1.0
0.8
0.6
0.4
SIT
STAND
H
conductance
P condition = 0,76
P group < 0,01
P group*condition= 0,75
cm/s/mmHg
SIT
WALK15
I
conductance
P condition = 0,14
P group < 0,01
P group*condition= 0,17
cm/s/mmHg
SIT
WALK30

Figure 2.3

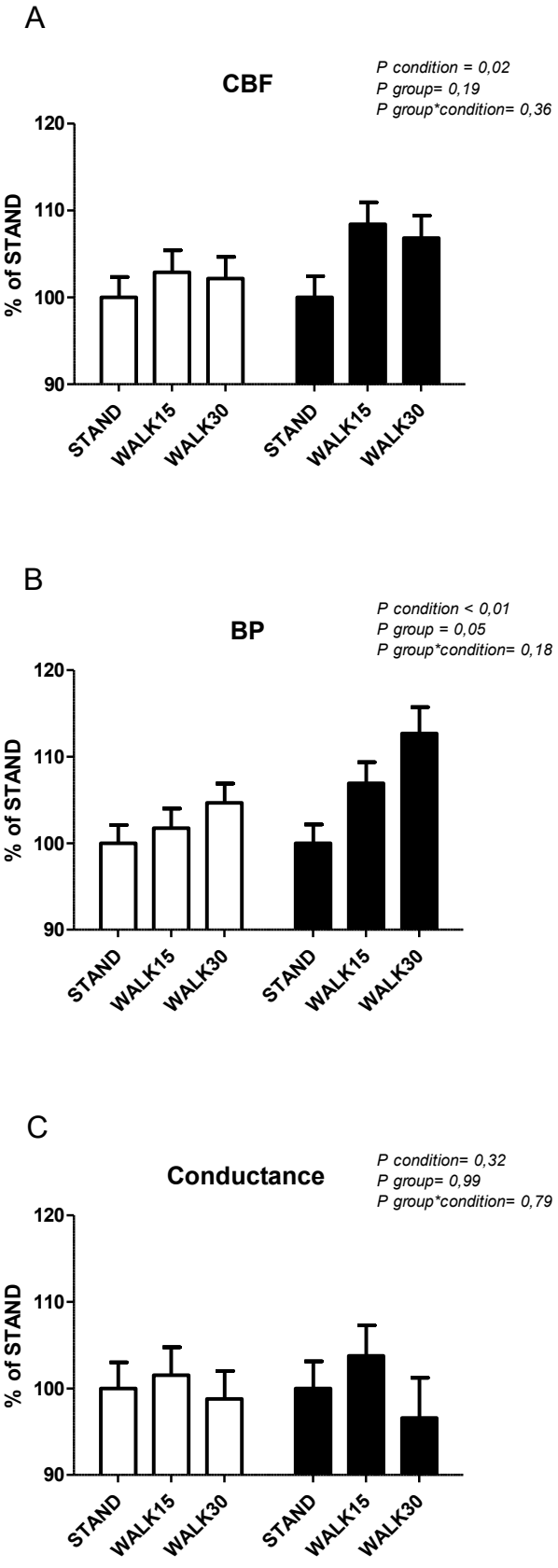

A
CBF
P condition = 0,02
P group= 0,19
P group*condition= 0,36
% of STAND
120
110
100
90
STAND
WALK15
WALK30
STAND
WALK15
WALK30
B
BP
P condition < 0,01
P group = 0,05
P group*condition= 0,18
% of STAND
120
110
100
90
STAND
WALK15
WALK30
STAND
WALK15
WALK30
C
Conductance
P condition= 0,32
P group= 0,99
P group*condition= 0,79
% of STAND
120
110
100
90
STAND
WALK15
WALK30
STAND
WALK15
WALK30

Table 2.1: Anthropometric characteristics of groups with result of MoCa Score. YG= young group, AG= age group. Values are mean ± SD.

	YG n (15)	AG n (15)
GENDER	4 F, 11 M	8 F, 7 M
AGE	24.47 ± 2.22	64.36 ± 5.86
WEIGHT (KG)	73.93 ± 11.61	81.50 ± 11.57
HEIGHT (CM)	178.73 ± 10.07	172.71 ± 11.27
BMI	22.99 ± 1.73	27.28 ± 2.60
MoCA SCORE	28.60 ± 1.08	28.14 ± 1.06

Experiment 3 - Figure captions

Figure 3.1 A) Carotid artery ultrasound alongside the cold pressor test (CPT). B) A healthy ultrasound image demonstrating wall tracking (yellow) used to calculate vessel diameter and C) Diameter of the carotid artery during both 1: Baseline measurement and 2: In response to the CPT. Demonstrating a healthy dilatory response. Adapted from (van Mil, Pouwels et al 2017).

Figure 3.2 Mean and Standard Deviation participants during the cold pressor test (CPT) **A**) Mean arterial pressure (MAP) response to the CPT and **B)** Diameter response to the CPT. One-Way ANOVA performed to compare baseline vs increase in both MAP and diameter. * denotes P= >0.05

Figure 3.1

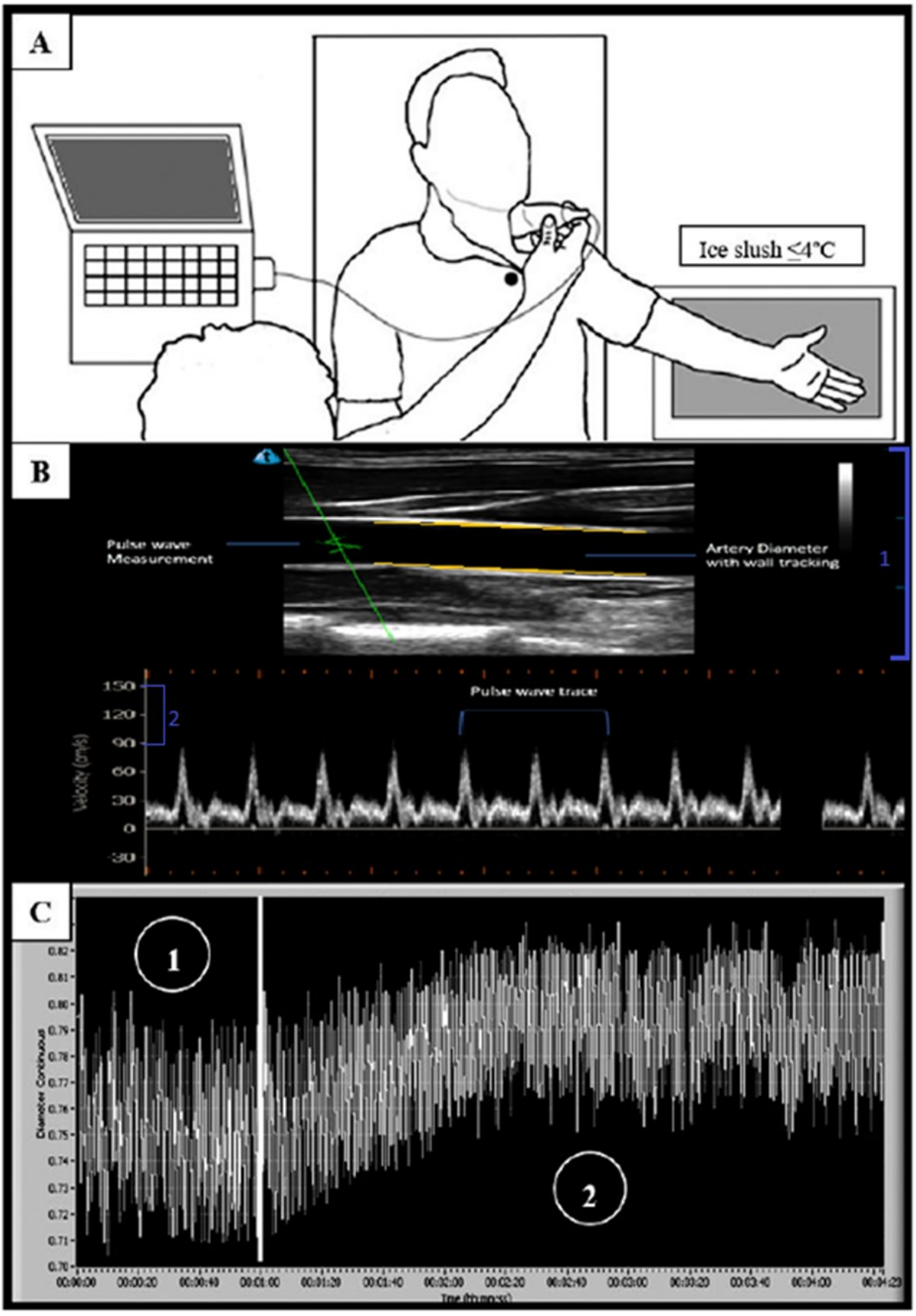
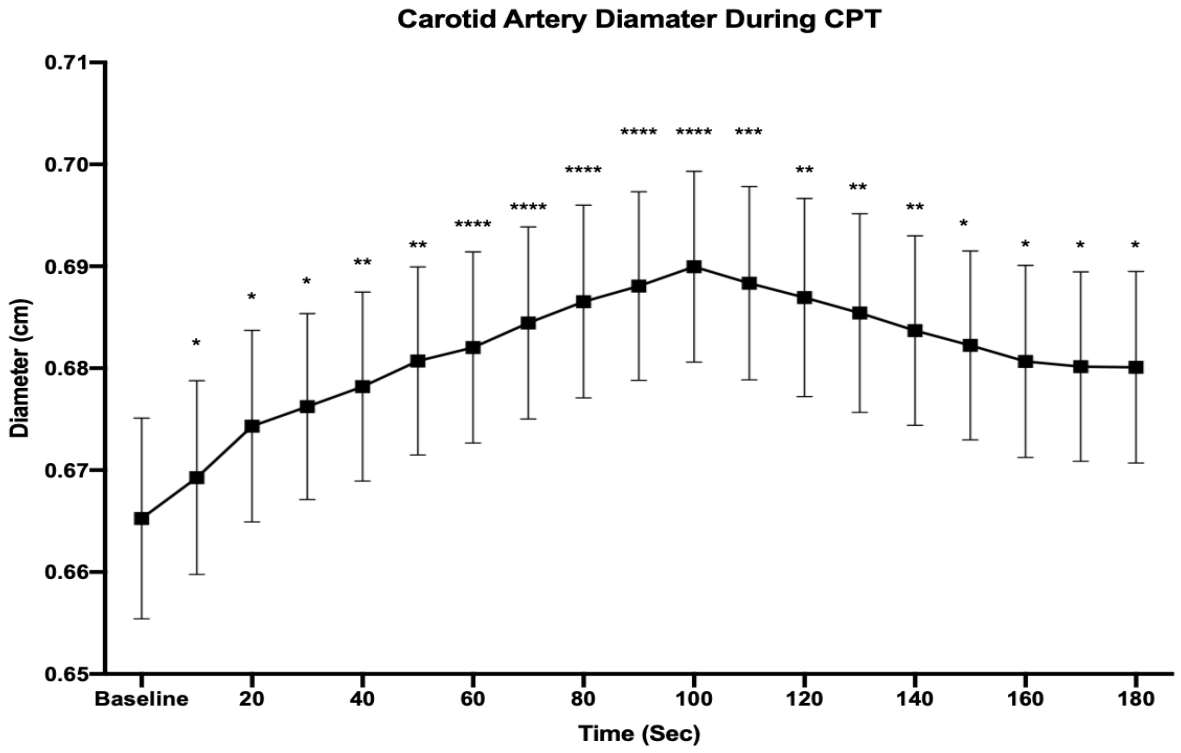

A
Ice slush ≤4°C
B
Pulse wave Measurement
Artery Diameter with wall tracking
1
Pulse wave trace
2
150
120
90
60
30
0
-30
Velocity (cm/s)
C
1
2
Diameter Continuous
0.82
0.81
0.80
0.79
0.78
0.77
0.76
0.75
0.74
0.73
0.72
0.71
0.70
00:00:00
00:00:20
00:00:40
00:01:00
00:01:20
00:01:40
00:02:00
00:02:20
00:02:40
00:03:00
00:03:20
00:03:40
00:04:00

Figure 3.2


Carotid Artery Diamater During CPT
Diameter (cm)
0.71
0.70
0.69
0.68
0.67
0.66
0.65
Baseline
20
40
60
80
100
120
140
160
180
Time (Sec)

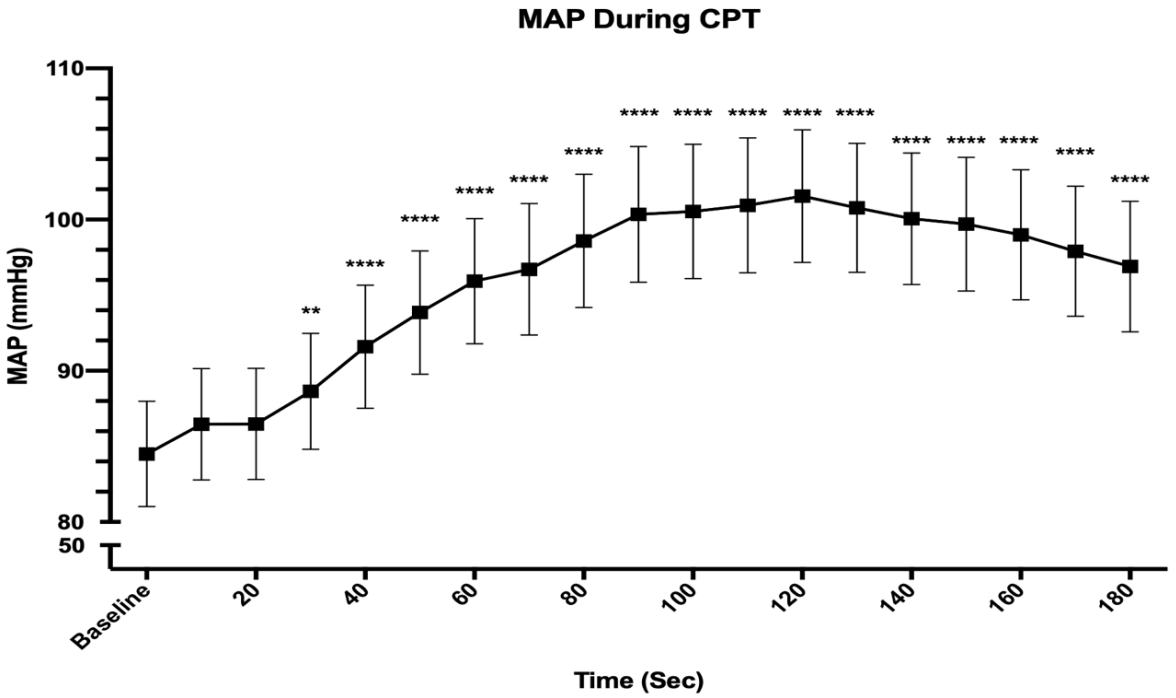

MAP During CPT
MAP (mmHg)
110
100
90
80
50
Baseline
20
40
60
80
100
120
140
160
180
Time (Sec)

Table 3.1 Correlation between CAR% and the blood pressure variables during CPT for all participants and the dilation group. ΔBP is the absolute difference between peak and baseline BP, whereas relative ΔBP is the percent increase from baseline to the peak BP.

A: BP Change defined as low (<15%), medium (15-30%) and high (>30%)

	All participants		Dilation group (N=34)	
	Pearson correlation	*p-value*	*Pearson correlation*	*p-value*
Baseline diameter	-0.354	0.025	-0.588	<0.001
Baseline MAP	0.209	0.197	0.298	0.087
Peak MAP	0.359	0.023	0.422	0.013
ΔMAP	0.326	0.040	0.327	0.059
Relative ΔBP	0.272	0.089	0.226	0.199
MAP change$_A$	0.331	0.037	0.271	0.121

Table 3.2 CAR and blood pressure results of the groups divided based on relative ΔMAP (low= <15%, medium = between 15% and 30%, high = >30%). Relative ΔMAP is the percent increase from baseline to the peak MAP.

	Low relative ΔBP (n=8)	Medium relative ΔBP (n=20)	High relative ΔBP (n=12)	p-value
Age	42.6 ± 9.3	40.9 ± 9.2	44.3 ± 10.6	0.613
Weight (kg)	83.5 ± 12.3	81.2 ± 11.7	83.9 ± 15.3	0.823
Height (m)	1.76 ± 0.08	1.77 ± 0.07	1.79 ± 0.07	0.599
BMI (kg/m^2)	26.7 ± 2.2	26.0 ± 3.5	26.1 ± 4.6	0.889
Positive family history	1.5 ± 0.76	1.2 ± 0.8	1.3 ± 0.8	0.664
Baseline diameter (cm)	0.67 ± 0.05	0.65 ± 0.05	0.69 ± 0.08	0.219
CAR%	1.3 ± 4.6	4.5 ± 4.5	6.4 ± 6.5	0.109
Baseline MAP (mmHg)	95 ± 11	85 ± 8	90 ± 9	0.053
Peak MAP(mmHg)	102 ± 13	106 ± 10	125 ± 15[ab]	<0.001
Relative ΔMAP (%)	7.5 ± 4.3	23.7 ± 3.9 [a]	38.0 ± 4.0[ab]	<0.001

[a] *Post-hoc significantly different from group 1*
[b] *Post-hoc significantly different from group 2.*

Table 3.3 Participant characteristics and CAR% when divided into groups based on the presence of diameter dilation or vasoconstriction. P-values refer to an unpaired t-test.

	Dilator (34)	Constrictor (6)	p-value
Age	42.08±9.3	43.17±12	0.904
Weight (kg)	81.05±11.2	90.3±18.7	0.343
Height (m)	1.779±0.072	1.750±0.050	0.648
BMI (kg/m^2)	25.58±2.9	29.37±5.3	0.060
Number of risk factors	0.441±0.504	0.167±0.408	0.372
Baseline diameter (cm)	0.67±0.054	0.66±0.104	0.430
CAR%	5.7±4.7	-2.9±2.48	<0.001
Baseline MAP (mmHg)	88.7±7.4	91.5±8	0.464
Peak MAP (mmHg)	110±15	109±19	0.288
Relative ΔMAP (%)	22.3±10	20±15	0.518