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Evidence of vascular function plasticity induced by small muscle training

Tutor: Prof. Fabio Esposito

Angela Valentina Bisconti
Matricola: R11223

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"Very many maintain that
all we know is still infinitely less than all that still remains
unknown. . . ."

William Harvey

Grazie

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Chapter 1- Introduction and Overview

According to the American Heart Association and the World Health Organization, cardiovascular disease is the primary cause of death, with an estimated 17.7 million people died in 2015, representing 31% of global deaths (Mendis et al. 2011; Mosca et al. 2011; Benjamin et al. 2017). Interestingly, the female incidence of mortality due to cardiovascular events has clearly exceeded that of male since 1984 (Mosca et al. 2011).

By addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol, most of the cardiovascular diseases can be prevented (Mendis et al. 2011; Mosca et al. 2011; Benjamin et al. 2017).

Physical exercise is the most important non-pharmacological treatment leading to several cardiovascular protective effects (Benjamin et al. 2017) via a direct impact on the vasculature and on the autonomic response (Green et al. 2008; Joyner and Green 2009). Specifically, several retrospective studies shown that physical exercise has an indirect capacity to prevent or reduce cardiovascular risk factors, such as blood pressure, lipid profile, insulin resistance, and obesity. Additionally, physical exercise directly impacts on cells and tissues of the heart and circulatory system (Thompson et al. 2003), hence improving vascular functionality inducing functional and structural arterial adaptations (Green et al. 2017b).

In the modern society, not everyone could practice physical activity on a daily basis, because of several limitations due to urbanization, such as high-density traffic, pollution; lack of parks, sidewalks, sport/recreation facilities or more simply, particular diseases in which the traditional physical exercise approach results unfeasible. All together, these factors make difficult to increase physical activity in daily life, and discourage people from becoming more active.

Therefore, to date one of the main questions among the researches is how to improve/maintain proper physiological functions and health especially in disease individuals by utilizing the proper exercise-based rehabilitation approach, in order to not only reducing cardiovascular mortality and decreasing the hospital admissions, but also improving their quality of life (Anderson et al. 2016).

Exercise-related improvements in vascular functionality are primarily attributed as shear stress-dependent mechanism (Tinken et al. 2009; Birk et al. 2012, 2013, Green et al. 2017b, a). Shear rate is the laminar shear force running in parallel to vessels long axis (Niebauer and Cooke 1996). During exercise, changes in blood flow and hemodynamic cause also a change in shear rate lastly stimulating endothelial cells to release vasoactive factors and leading to the idea that repeated exercise sessions have positive and beneficial effect on the overall endothelium health (RS et al. 2015; Davies 2016a; Green et al. 2017b, a).

In order to meet the increased metabolic demand and in accordance to exercise intensity, a markedly increase in blood flow and shear stress in active regions occurs at the onset of exercise (Green et al. 2005; Thijssen et al. 2009a). Specifically, it seems that the amount of muscles involved in the exercise could generated different shear and blood flow pattern (Green et al. 2005; Thijssen et al. 2009b, a; Tinken et al. 2009). For example, handgrip exercise, a small muscle exercise, induces a large hyperemic response in brachial artery due to a downstream resistance vessels vasodilation causing only minor changes in blood pressure and cardiac output (Green et al. 2005). On the contrary, lower limb exercise (i.e. cycling), a whole body muscle exercise, determines a hyperemic response in the femoral artery due to changes in downstream resistance vessel dilation in concert with the increases in central driving pressure (Green et al. 2016).

Therefore, local vasodilator mechanisms along with increases in arterial pressure and cardiac output contribute to exercise hyperemia, leading to significant increases in shear stress in active areas during exercise. Moreover, other evidences suggest that the improvements in brachial artery vascular function, despite the lack of forearm exercise, may be widespread and not specific to the vascular bed of the trained skeletal muscle (Birk et al. 2012).

Respiratory muscles being part of the musculoskeletal system can be trained (Esposito et al. 2010b, a). Because of this, it is possible that respiratory muscle training (RMT) could be used as an alternative exercise paradigm causing alteration in the peripheral hemodynamic, including no exercising areas, to improve vascular health. Previous evidences proved that RMT has a primary positive effect in static and dynamic lung volumes, together with maximal inspiratory pressure (Esposito et al. 2010b, a), and a secondary important effect in increase cardiac vagal tone, lastly affecting the autonomic nervous system balance (Hepburn et al. 2005). To date, the effects of RMT on the overall vasomotor response (Thijssen et al. 2011a; Green et al. 2016) (a well-recognized marker of cardiovascular health) has not been investigated yet.

Another small muscle exercise modality is the dynamic knee extension. This type of exercise was used the first time by Andersen et al., in 1985, and it has the advantage that the work is performed almost exclusively by one muscle synergy (i.e. quadriceps muscle) (Andersen et al. 1985). This isolated quadriceps exercise could cause pronounced elevation not only in local energy turnover but also in whole-body oxygen uptake and related variables (Andersen et al. 1985). More recently, this exercise model was used to training a chronic heart failure population (Esposito et al. 2010c, 2011). This type of population is mainly characterized by central limitations leading to a higher exercise intolerance. Eight weeks of training produced clear improvements in muscle structure, peripheral convective and diffusive oxygen transport, and

subsequently, oxygen utilization supporting the efficacy of local skeletal muscle training (Esposito et al. 2011), and begging the question if positive effect could be detected also in the vascular functionality.

The ability of the vessels to alter their diameter (i.e. vasodilation or vasoconstriction) to maintain the homeostasis of the vascular tone, ensuring that the blood flow matches the demand of the skeletal muscles and other organs, both at rest and during exercise is defined vasomotor response. Extrinsic factor, such as the autonomic control of the sympathetic neural drive (global control) (Wallin and Charkoudian 2007; Sandoo et al. 2010; Bruno et al. 2012), and intrinsic factor such as the capacity of the endothelial cells to respond to mechanical stress by releasing vasoactive molecules (i.e. nitric oxide, NO) (Furchgott and Zawadzki 1980; Furchgott and Vanhoutte 1989; Furchgott 1990; Furchgott 1991; Sandoo et al. 2010) together with other possible factors, such as pH and temperature, interact to determines the prevalence of a vasoconstriction or vasodilator effect on the arterial wall. Therefore, autonomic and/or an endothelium dysfunction could lead to an impaired vasodilatory response (Green et al. 2008; Joyner and Green 2009; Sandoo et al. 2010)

The concept of "sympatho-vagal balance" reflects the autonomic state resulting from the sympathetic and parasympathetic influences (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996), and could be considered as the global control branch of the vasomotor response. The most common technic used non-invasively and in vivo is the heart rate variability (HRV) (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996). HRV is the physiological phenomenon of variation in the time interval between heartbeats, in which the low frequency (LF) band of HRV mainly reflects the sympathetic component, while

the high frequencies (HF) are more closely related to vagal activation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996). There are several factors that could affect the sympatho-vagal balance, such as circulating hormones, modifications in heart rate and/or breath frequency, presence of pathologies, and even exercise training programs (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996). In detail, endurance exercise training lead to an increase in the HF, indices of vagal activation, lastly reducing the balance between LF and HF at rest, during, and immediately after exercise (Bellenger et al. 2016).

Intrinsic factor, also qualify as peripheral component, of the vasomotor response, could be also assessed non-invasively and in vivo by flow mediated dilation (FMD) technique (Celermajer et al. 1994; Thijssen et al. 2011a). FMD is commonly used to assess endothelial function, and it determines the dilatator capacity of the artery in response to a hyperemic-induced shear stress stimulus (Celermajer et al. 1994; Thijssen et al. 2011a). Indeed, FMD generates a hyperemic response, which is sensitive to and strictly dependent on the frictional or drag force apply on the inner vessels lay representing the shear stress (Pyke and Tschakovsky 2005). Therefore, either the vasodilatation and the magnitude of the stimulus imposed are considered the main outcomes of the FMD. It has been also well documented that long term exercise training lead to enhancements in FMD as meaning that improvements in the endothelial functionality occurred (Birk et al. 2012; Green et al. 2017b, a).

Passive limb movement (PLM) has been recently proposed as another non-invasive approach to assess the NO-dependent endothelial function (Trinity et al. 2012). This new maneuver, consisting in a rapidly and passive knee flex- extension realized by an external operator,

provokes a hyperemic response in the peripheral circulation without the presence of any increase in metabolism likely occurring during exercise (Trinity et al. 2012). It was also demonstrated that PLM response, rather than that induced by FMD, is primarily mediated by NO, therefore a useful tool to better assess endothelium functionality (McDaniel et al. 2010b; Trinity et al. 2012; Rossman et al. 2016). However, to date no data are available regarding the effects of exercise training on PLM response.

Thus, the aim of this dissertation was to evaluate the effects of two different types of small muscle exercise training on the peripheral vasomotor response in young healthy people. Two studies were developed in which the purposes were:

1) to evaluate the effects of eight weeks of RMT on both central (i.e. the balance between the sympathetic and parasympathetic neural system assessed by heart rate variability) and peripheral (i.e. the ability of the endothelium to release NO causing vasodilatation assessed by FMD) components of vasomotor response, in young healthy females;

2) to evaluate the effects of single-leg knee extension training (KE) on vasomotor response in the lower limb directly involved with exercise (i.e. femoral artery) and on the upper limb, not involved with KE (i.e. brachial artery).

We hypothesized that (i) RMT could improve FMD in the brachial artery (beneficial effect on peripheral control due to systemic factors influenced by exercise training) via a reduction in sympathetic drive (central control), and that (ii) KE could raise the peripheral blood flow also in limb non-directly involved in the exercise leading to positive effects in both exercised and not exercised limbs.

Chapter 2- Vascular Function

2.1 Regulation of vascular tone and blood flow in skeletal muscles

Prerequisite of life is the oxygen delivery to cells. Oxygen delivery bridges gap between oxygen from the outside airspace to the interstitial space around cells. This oxygen cascade occurs due to convection and diffusion steps involving the lungs and the cardio-circulatory system. The macro and micro vasculature together with the heart are the main structure and function determinants of the cardio-circulatory system: the heart has the main role to intermittently pump the blood through the ventricular ejection; the large arteries, due to their viscoelastic properties, smooth the high pulsatile pressure and flow out; the microvasculature mediates steadily the delivery of oxygen and nutrients to the tissues.

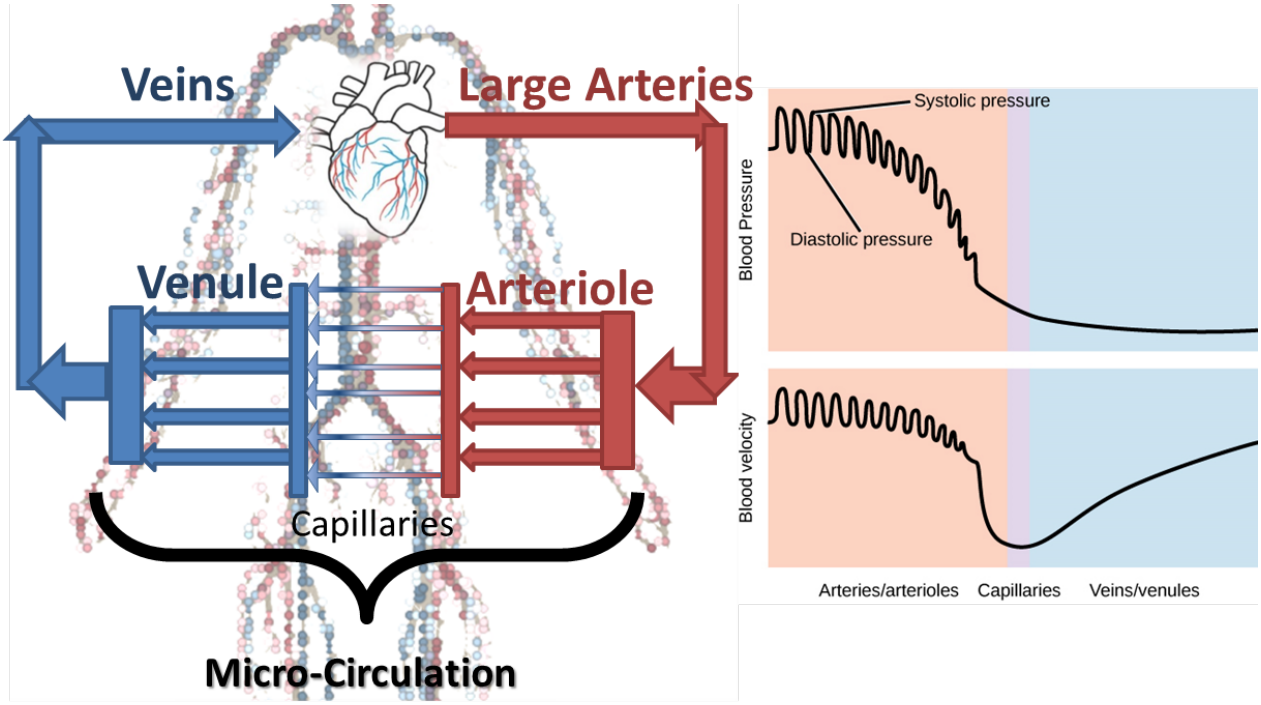

Figure 1. Chart of the circulatory system (on the right). Blood pressure component and velocity throughout the blood vessels (on the left). Blood pressure includes systolic, diastolic and pulse pressure.

As shown in Figure 1, the vasculature, starting from the aorta, can be anatomically and functionally classified into:

1. Large and medium arteries;
2. Smaller feed arteries and terminal arterioles, or precapillary resistance arterioles;
3. Capillaries, or exchange vessels (without any contractile elements);
4. Venules, or post-capillary resistance vessels;
5. Large veins or more voluminous venous capacitance.

The blood flow running from the arterioles to the venules passes through a variable number of capillaries, this system is recognized as micro-circulation. The micro-circulation, regulated by the arteriolar resistance and capillary recruitment, allows restraining to the exchange area between blood and parenchymal cells. This phenomenon, predominantly occurring in heart and skeletal muscle and lungs, is intended to better regulate the amount of blood flow, and therefore oxygen and nutriments, where needed.

2.1.1 Microvascular Unit: Anatomy

The microvascular unit is defined as a terminal arteriole and the group of capillaries it supplies. The microvascular unit represents the smallest functional unit for blood flow regulation in skeletal muscle (Korthuis 2011a; Shimokawa and Satoh 2014; Rizzoni et al. 2015; Jacob et al. 2016) (Figure 2). Based on the position on the arterial tree, arteries can be divided into conducting arteries, conduit arteries and resistance arteries. Within the microcirculation, it is possible control the distribution and the magnitude of blood flow to the tissues because of the interaction between arteriolar, capillary, and venular segments, according to local and regional metabolic demand (Mellander and Johansson 1968; Korthuis 2011a; Rizzoni et al. 2015; Jacob et al. 2016). Blood flow reaches the muscle primarily through large and medium arteries that

are distributed along the long axis of the muscle. Due to the high pulse pressure, the conducting arteries have a thicker wall containing large amount of elastic tissues compare to all the other vessels. The main role of conducting arteries is to dampen out the oscillatory changes in blood pressure resulting in therefore called intermittent ventricular contraction. Through this process, large arteries give rise to feed arteries that course toward the muscle at oblique angles in respect to the large ones (Figure 2). Feed arteries, or conduit arteries, are really important for the control of blood flow, which account for 30 – 50 % of the total resistance in the blood stream. Anatomically, they are proximal to the terminal arterioles and capillaries that are embedded in the skeletal muscle tissue (Figure 2). Arterioles, also known as resistance arteries, running perpendicular to the skeletal muscle fiber, enter in the perimysium and spread into numerous capillaries, which travel parallel to the muscle fiber and embedding the endomysium (Figure 2). The main role of these resistance arteries is to adequately perfuse the organ tissues. The terminal arterioles are the last branches to contain vascular smooth muscle, explaining why the group of capillaries perfused by a terminal arteriole has been termed the microvascular unit. Smooth muscle cells, highly innervated by the sympathetic nerve, allow the arterioles to dilating or constricting in response to sympathetic (de)activation (Rhodin 1980). Laminar blood flow with its dragging frictional force (shear stress) is another possible stimulus causing vasodilation in arterioles (Galley and Webster 2004; Sandoo et al. 2010).

Capillaries are the site of tissue perfusion; indeed, the main function is to improve diffusion of nutrients, oxygen, metabolites etc. between blood flow and tissues. However, capillaries are non-uniform distributed around myofibrils accordingly with the fact that muscle fiber circumference is quite variable. This inhomogeneous distribution indicates that also the oxygen is differently delivered, even under condition of maximal capillary recruitment (Bagher and

Segal; Segal 2005; Sarelius and Pohl 2010). Additionally, comparison between capillaries network anatomy in red and white muscle fiber showed that their density, and number of interconnections among them are greater in oxidative muscle fibers (Folkow and Halicka 1968; Andersen 1975).

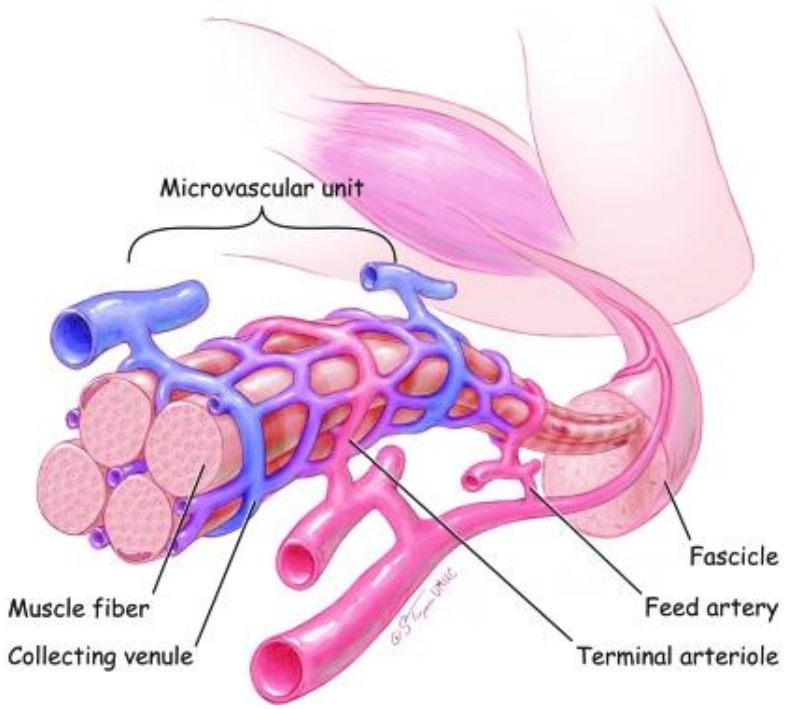

Figure 2. General anatomical representation of the microvascular unit and the muscle fibers it supplied, from Korthuis RJ, *Skeletal Muscle Circulation,* (Morgan & Claypool Life Sciences, 2011) Chapter 2.

The arrangement of venules and veins is similar to that described for the arterioles and arteries, indeed the main arterioles and venules are paired. On the contrary, the outflow blood through a given venule is not derived from its parallel venule, but rather arises from arterioles some distance away (Figure 2).

2.1.2 Vasomotor Response and its controlling factors

As describe above, the microvascular unit is in charge to control the peripheral circulation accordingly to the metabolic demand. The ability of the vessels to alter their diameter is commonly defined as vasomotor response. More specifically, it can refer to vasodilator and vasoconstriction actions. Under rest condition, basal diameter of arterial and venous vessels depends on the degree of smooth muscle cells contraction, which in turn could enhance or reduce the vascular tone (Rhodin 1980). Basal vascular tone is different among organs. There are organs with a large vasodilatory capacity together with high vascular tone (e.g. skeletal muscle, skin, splanchnic circulation and myocardium), and organs with low vasodilatory capacity and low vascular tone (e.g. renal circulation). However, vascular tone is affected by competing vasodilator and vasoconstrictor factors. It is possible recognized:

- Extrinsic factors, which origin is outside the organ or tissues in which the blood vessel is located; their function is to regulate arterial blood pressure by altering systemic vascular resistance due to the release of extrinsic factor, such as products of sympathetic nerves and/or angiotensin II, primarily mediating by an increment in the vascular tone (vasoconstriction);
- Intrinsic factor, which origin from the vessel itself or surrounding tissues; their function is essential for local blood flow regulation. Intrinsic factors include myogenic mechanism (vasoconstriction), endothelial factors such as nitric oxide (vasodilation) and endothelin (vasoconstriction), metabolites, or hypoxia (vasoconstriction).

All these mechanisms, causing either constriction or relaxation in blood vessels, are related to different transduction mechanisms that ultimately influence the interaction between actin and myosin in the smooth muscle cells.

Interestingly, in 1970, Prof. Duling, was the first to introduce the concept of "propagated vasodilatation", describing the spread of vasodilatation along the hamster cheek pouch microvascular unit in response to an appropriate stimulus (Duling and Berne 1970). Specifically, it was shown that acetylcholine, applied using a microinophoresis as a mean of achieving relatively precise temporal and spatial application of drugs to single arterioles, caused a propagated vasodilation in the peripheral vasculature explaining the integrative relationship between terminal arterioles and larger vessels (Duling and Berne 1970).

A modern idea for the same concept was given by Segal in 2015 (Segal 2015), that renamed and fully explained this phenomenon as "ascending vasodilation" (Figure 3).

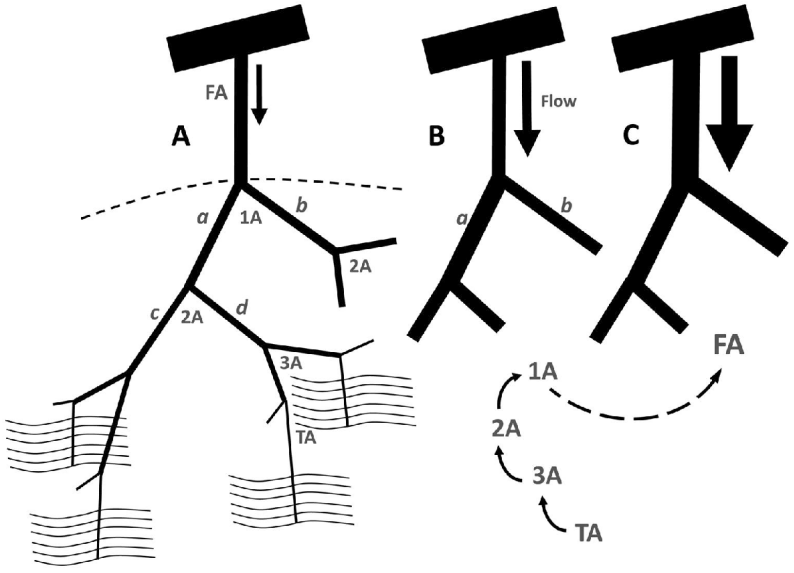

Figure 3 "Ascending vasodilation in a microvasculature resistance network", from Segal, *Integration and modulation of intercellular signaling underlying blood flow control,* (J Vasc Res. Available in PMC 2016). Illustration describes a feed artery (FA) ascending from a large conduit artery. The FA brings blood into the tissue (e.g., skeletal muscle; dotted line) and gives rise to primary (1A) arterioles, *a* and *b*. These 1A's each give rise to second order (2A) arterioles, which branch into third-order (3A) and then terminal (TA) arterioles, with each TA giving rise to groups of capillaries (post-capillary micro vessels omitted for clarity). B. Vasodilation originating in 3A and 2A (for branches *c* and *d*) "ascends" into the parent 1A (branch *a*) but not into the proximal FA. At the same time, branch *b* may receive little or no dilator signal from its daughter 2A and 3A (not shown) supplying an inactive region of the muscle. Under these conditions, lack of ascending vasodilation into FA limits tissue blood flow. C. With greater dilation of its daughter arterioles, vasodilation ascends into branch *b* which, together with signals along branch *a* are summed in the FA. Dilation of the FA reduces the proximal resistance restricting tissue blood flow. Total blood flow into the microcirculation increases dramatically.

The ascending vascular dilatation, shown in Figure 3, highlighted that when vasodilation is primarily originating in distal micro vessels, it is, in turn, spread upstream into the proximal feed artery and other supply arteries in order to provided maximal blood flow in that area (Segal and Duling 1986). On contrary, when ascending vascular dilatation do not occur any constriction of feed arteries upstream from arteriolar networks negatively affects the microcirculation causing a restriction in a blood flow, even when arterioles within the tissue are maximally dilated (Segal and Duling 1986; Segal and Jacobs 2001; Segal 2005). It results clear that differences within resistance networks in the vasomotor control is a mechanism for extent or reduce the capillary surface area for oxygen extraction especially when microvascular blood streaming is limited by proximal vasoconstriction (Segal 2005, 2015). A pivotal role in the vasomotor response and relative ascending vasodilation is played by the functional interaction between skeletal muscle fibers and respective vascular smooth muscle and endothelial cells, and neural projection.

Specifically, the endothelium can be considered as a dynamic organ which controls the entire vascular system (Galley and Webster 2004). The integrity of the endothelial cells structure and function are important to maintain vessels wall and circulatory function due to their metabolic and synthetic function (Galley and Webster 2004). They are located on the intima and exert autocrine, paracrine and endocrine actions by responding to various hormones, neurotransmitters and vasoactive factor (lastly affecting vasomotor response) (Galley and Webster 2004). Endothelium is essential for body homeostasis, indeed, disease processes, such as atherosclerosis, hypertension, pulmonary hypertension, sepsis and inflammatory syndromes, have unrestrained endothelial cell response, lastly causing endothelial injury, dysfunction and activation. Endothelial cells, as cited above, are all located in the intima, but different type of

vessels could display different phenotype and structure of them (e.g. in arteries and veins endothelial cells appear thicker than those in capillaries, thinner and fenestrated, to better allow exchange with the blood flow) (Galley and Webster 2004). Furthermore, not only the same stimulus could generate heterogeneous endothelial response in different vascular bed, but also different endothelial response in the same vascular section (Galley and Webster 2004).

As above mentioned, the endothelium releases various vasoactive factors, among the most important there are: nitric oxide, endothelin-1 and endothelium-derived hyperpolarizing factor. Particularly attention is also given to sympathetic neural activation main extrinsic factor in controlling the vasomotor response.

2.1.2.1 Nitric Oxide

Nitric Oxide (NO) is a free radical gas and an endogenous vasodilator (endothelium-dependent) that regulates blood circulation in the human body. NO was identified for the first time in 1980 by the Nobel Prize Furchgott and Zawadzki (Furchgott and Zawadzki 1980) and its main role is to maintain basal vessel tone (Burke; Vallance et al. 1989; Furchgott 1990; Sandoo et al. 2010). L-arginine is the only amino acid that generates significant amounts of NO in the human body[21,23]. To catalyze L-arginine in NO a nitric oxide synthase (NOS) is required. Specifically, three forms of NOS exist: neural isoform (nNOS), it is involved in synaptic transmission regulating neuro-transmitter release (Michel and Feron 1997); macrophage or inducible isoform (iNOS) generates extraordinarily high concentrations of NO, in part to kill bacteria and in response to an injury or infectious process. iNOS is produced only in cells that have been exposed for a relative long time to inflammatory mediator as well as other type of injury stimuli that activate macrophages (Michel and Feron 1997). Lastly, endothelial NOS (eNOS) that produces NO in the vasculature (Michel and Feron 1997).

A schematic representation of eNOS activity and its role in regulate vessels blood flow is given in Figure 4 (Zhu et al. 2016). In detail, inactive eNOS is bound to a specific protein called caveolin, which is located in small invaginations into the cell membrane called caveolae (Michel and Feron 1997). eNOS disconnects from caveolin and becomes active when intracellular concentration of Ca^{2+} dramatically increase (Michel and Feron 1997). However, there are also others NO agonists, such as bradykinin (BK), acetylcholine (ACh), adenosine triphosphate (ATP), adenosine diphosphate (ADP), substance P and thrombin, that can influence the release of Ca^{2+} from the endoplasmic reticulum allowing the detachment of eNOS from caveolin (Michel and Feron 1997). Once intracellular Ca^{2+} stores are completely depleted a signal is sent to the membrane receptors to open Ca^{2+} channels allowing extracellular Ca^{2+} to enter into the cell (Michel and Feron 1997).

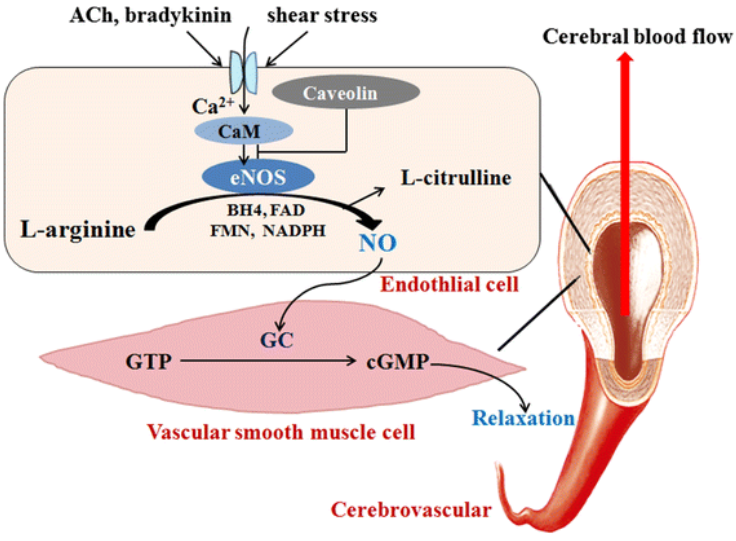

Figure 4 Endothelial nitric oxide production and its action on smooth muscle cells in a representative cerebral artery, from Zhu J, *Endothelial nitric oxide synthase: a potential therapeutic target for cerebrovascular disease*, (Molecular Brain in BCM 2016). ACh= acetylcholine; BK= bradykinin; CaM= calmodulin; BH4= tetrahydrobiopterin; FAD= flavin adenine dinucleotide; FMN = flavin mononucleotide; NO= nitric oxide; GC= soluble guanylyl cyclase; guanosine triphosphate =GTP; cGMP= cyclic guanosine-3', 5-monophosphate.

At this point, Ca^{2+} binds the protein calmodulin (CaM) in the cytoplasm of the cell and, due to its new structural change, attaches eNOS (Putney 1986). eNOS converts L-arginine into NO (Palmer et al. 1988) as shown in Figure 4. It is important to highlight that this mechanism of NO production is not only dependent on the levels of intracellular/extracellular Ca^{2+}, but also to the level and activity of tetrahydrobiopterin (BH4) (Davignon and Ganz 2004; Crabtree et al. 2011). Specifically, diseases state, matched by vascular dysfunction, present a significantly reduction in NO bioavailability and activity, together with an increase in oxidative stress (Crabtree et al. 2011). In this scenario, Bh4 seems to be essential because of its role in "coupling" eNOS and lastly maintain efficient endothelial function (Vásquez-Vivar et al. 1998; Kojda and Harrison 1999; Crabtree et al. 2011). Indeed, the availability of BH4 determines the balance between NO and superoxide production. Oxidation of BH4 forms 7,8-dihydrobiopterin (BH2), which is inactive for NOS function and can compete with BH4 for NOS binding(Vásquez-Vivar et al. 1998; Kojda and Harrison 1999; Crabtree et al. 2011). BH4 and BH2 bind eNOS with similar affinity but the increment in the BH2 bound causes eNOS uncoupling and superoxide production (Vásquez-Vivar et al. 1998; Kojda and Harrison 1999; Crabtree et al. 2011).

Another important mechanism to produce NO is the phosphorylation of eNOS (Butt et al. 2000). This pathway occurs when intracellular Ca^{2+} starts to decrease. The phosphorylation of eNOS by cyclic guanosine-3', 5-monophosphate (cGMP)- and cyclic adenosine monophosphate (cAMP)-dependent protein kinases not only stimulates Ca^{2+}/CaM-dependent eNOS activity, but also causes a Ca^{2+}-independent partial activation of eNOS.

Partially related to this mechanism is the production of NO due to the shear stress stimulus. Shear stress, in vascular rheology, is defined as frictional force of the blood flow on the

endothelial layer. Shear stress is opposed by tension and deformation in the endothelium. On the other hand, circumferential distension of blood pressure is opposed by circumferential stress and strain (stretch) in the vessel wall (intima, media and adventitia). An increase in shear stress determines an increase in NO production via not only eNOS phosphorylation, but also through an endothelial-receptors stimulation moving Ca^{2+} and K^{+} ions across the endothelial cell membrane to ultimately increase intracellular Ca^{2+} (Tran et al. 2000; Boo et al. 2002). Duration of the shear stress determines the contribution of Ca^{2+} and eNOS phosphorylation for NO production (Tran et al. 2000; Boo et al. 2002). Specifically, short exposure to shear stress causes an intracellular Ca^{2+} release. On the contrary, when the shear stress stimulus lasts longer than 30 minutes intracellular Ca^{2+} stores are depleted, and NO production is more dependent on eNOS phosphorylation (Tran et al. 2000; Boo et al. 2002). After synthesis, NO diffuses across the endothelial into the smooth muscle cells (Figure 4). NO, upon binding the enzyme soluble guanylyl cyclase (GC), enables the conversion rate of guanosine triphosphate (GTP) to cGMP determining the relaxation of smooth muscle cells (Ignarro et al. 1986).

The mechanisms described above interact among each other to maintain the NO production constant, and then regulate vascular basal tone.

2.1.2.2 Endothelin-1

Endothelin (ET) is a potent endogenous vasoconstrictor factor and it is present in three different isoforms in the body. ET isoform is known as ET-1 or preproendothelin-1: endothelial cells convert preproendothelin to proendothelin, and then to mature endothelin, which the endothelial cells release causing vasoconstriction on the smooth muscle cell (Yanagisawa et al. 1988).

Among the factors causing the ET-1 release are low shear stress, free radicals level and inflammatory state (inflammatory marker like interleukins and TNF-α). Among the factors

inhibiting ET-1 are high shear rate and NO (Kowalczyk et al. 2015). Shear stress could cause a decrease in ET-1 expression, after initially promoting it, and this is related to the type of activated receptors (Kowalczyk et al. 2015). Primarily, there are two types of ET receptor: ETA and ETB receptors found in the cardiovascular tissue belong to the G protein-coupled receptors family. Located in smooth muscle cells, ETA receptors are responsible for vasoconstriction, cell proliferation and pro-inflammatory effect (Kowalczyk et al. 2015). In the ETB receptor, two subtypes are recognized: ETB_1, which is expressed in endothelial cells and evokes NO-mediated vasodilation, and ETB_2, present in smooth muscle cells causing vasoconstriction. Indeed, when ET-1 binds ETB_2 as well as ETA receptors, smooth muscle Ca^{2+} channels open allowing the entering of the extracellular Ca^{2+}. On contrary, ETB_1-receptors could release vasodilatory factors such as prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor when stimulated (Kowalczyk et al. 2015). It is important to highlight that endothelial cell ETB_1 receptors are downregulated, while smooth muscle cell ETB_2 receptors are upregulated, thus enhancing vasoconstriction signal (Felix Böhm, Gunvor Ahlborg, Bo-Lennart Johansson 2002).

2.1.2.3 Endothelium-derived hyperpolarizing factor

Endothelium-derived hyperpolarizing factor (EDHF) is a non-specific vasodilator that hyperpolarizes the smooth muscle cells by making the membrane potential of the cell more negative (Ozkor and Quyyumi 2011). EDHF-response is due to several factors and it seems to primarily take place in the resistance vessels. Indeed, EDHF effect increases as the vessel size decreases working as a compensatory up-regulation of hyperpolarization when bioavailability of NO is reduced (Ozkor and Quyyumi 2011). When endothelial-cell agonist, such as bradykinin and/or Ach, stimulate endothelial cell, EDHF is released. As previously mentioned,

NO can dilate the vessels through the same pathway but for a shorter period before the present mechanism takes over. Indeed, when NO is inhibited, the hyperpolarization still occurs remaining non-NO mediated endothelium-dependent vasodilation partly attributed to EDHF (Ozkor and Quyyumi 2011). However, the exact pathway determining this hyperpolarization is still unknown, potential contributors are:

- Calcium-activated potassium (K_{Ca}^{2+}) channels;
- Epoxyeicosatrienoic acid (EETS)
- Hydrogen peroxide
- Gap Junction
- Potassium (K^{+})

2.1.2.4 Sympathetic Nervous System (SNS)

Vascular tone is affected by competing vasodilator and vasoconstrictor factors. Among the extrinsic factors, the autonomic nervous system, especially with one of the two divisions (the sympathetic branch), is known to play a central role in cardiovascular homeostasis (Wallin and Charkoudian 2007). SNS, releasing neurotransmitters (i.e. Endothelin-1), can control the vascular tone, mainly inducing vasoconstriction of the small resistance arteries (Wallin and Charkoudian 2007; Bruno et al. 2012). In detail, in order to maintain the vascular homeostasis in the short-term, the SNS rapidly regulates the vasomotor tone and the blood pressure in different physiological conditions by means of the classical autonomic reflexes (Wallin and Charkoudian 2007). It seems the SNS has also a long-term control in the blood pressure by inducing sustained blood pressure due to several mechanisms (e.g., by causing peripheral vasoconstriction, potentiating cardiac contraction, reducing venous capacitance, affecting renal sodium, and water excretion, through baroreflex dysfunction) (Joyner et al. 2008; Fink 2009).

Moreover, vascular functionality and sympathetic activity not only are key factor in development and prognosis of cardiovascular events and disease but also vascular homeostasis is maintained through activated pathways by the same signaling (i.e. NO, reactive oxygen species (ROS), endothelin (ET), the renin-angiotensin system) both at the autonomic nervous system level and in the vascular milieu leading to an integrated, multidistrict response (Bruno et al. 2012). Additionally, some studies reported that SNS could directly modulated functional and mechanical property of the arteries. Indeed, markers of vascular function are inversely related to various measures of sympathetic activation (Sverrisdóttir et al. 2010). An adrenergic hyperactivity is involved in the pathogenesis of several cardiovascular risk factors (Joyner et al. 2008), and interestingly this activation becomes chronic in several of the main cardiovascular disease leading to a vascular remodeling such as changes in vascular function and structure (Joyner and Green 2009). Thus, it seems that an autonomic dysfunction could indirectly induce vascular dysfunction and damage (Joyner et al. 2008; Joyner and Green 2009). Joyner MJ and Green JD, in a symposium review (2009), stated that the vicious cycle between autonomic dysfunction and endothelial dysfunction could be prevented or ameliorated by exercise training (Joyner and Green 2009). They also hypothesized that regular exercise and physical activity could have a direct protective effects against the premature development of cardiovascular disease, united by endothelial and autonomic dysfunction, by: i) improving the baroreflex response and central cardiovascular regulation due to an increase in the autonomic parasympathetic activity; ii) favoring vasodilatation and contributing to enhanced peripheral baroreflex by enhancing or maintaining an efficient endothelial functionality could limit age- or risk factor-associated with increases in vascular stiffness; iii) enhancing the positive

interactions between endothelial function and sympathetic outflow causing a limitation of the effects of high levels of baseline sympathetic outflow on blood pressure.

2.2 Techniques to assess vascular function

Quantifying the endothelium health state is really important. Not only it has a main role in maintaining the vasomotor response (Rhodin 1980; Galley and Webster 2004; Sandoo et al. 2010), but also the results obtained in the peripheral endothelial function assessments are positively correlated to those assessed in the coronary arteries (Anderson et al. 1995; Khan et al. 2008). Additionally, endothelial function is a well-recognized marker of cardiovascular health (Thijssen et al. 2011a). The endothelial health status is essential to recognize or prevent diseases and potential future cardiac events related to the loss of the endothelial functionality such as atherosclerosis, hypertension, metabolic syndrome, heart failure, autonomic dysfunction, etc (Thijssen et al. 2011a).

Endothelial function assessments are aimed to measure the vessels ability to dilate consequently to a stimulus. As describe above, endothelial cells respond to mechanical and chemical stimuli releasing vasoactive substances that cause smooth muscle cells relaxation. It is necessary to distinguish between endothelium or smooth muscle cells dysfunction, using endothelium-dependent and/or endothelium-independent vasodilatation assessments. Below, in Figure 5 are resumed all the main techniques used to assess endothelial function.

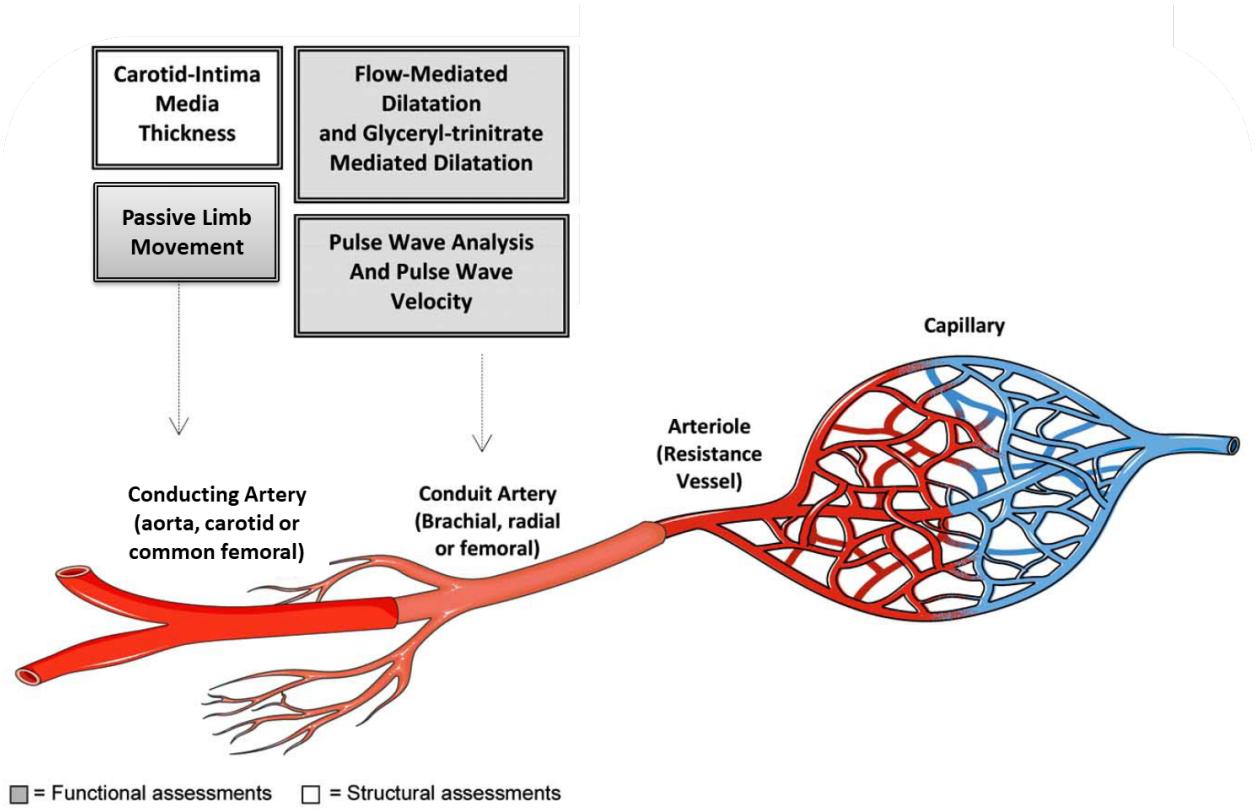

Figure 5. Schematic overview of the different assessments for endothelial health along the vascular bed, inspired to Sandoo A et al., *The endothelium and its role in regulating vascular tone*, (The Open Cardiovascular Medicine Journal, 2010).

2.2.1 Carotid intima-media thickness

Carotid- intima media thickness (cIMT) is a mean to qualify the structural health of the arteries. Pignoli, in 1986, was the first to propose a B-mode ultrasound system, as a useful non-invasive tool to measure intima media thickness in human in vivo (Pignoli et al. 1986). To date, cIMT together with plaque assessment methodology have been shown to be a predictor for cardiovascular events, even if the methodological variability accounts for differences in findings among the studies (Naqvi and Lee 2014). Following the ESC Council for Cardiology guidelines published in 2015, using a B-mode ultrasound system, it is possible to define the cIMT as a

double-line pattern visualized on both walls of the common carotid artery in a longitudinal section view at carotid bifurcation point (Iana et al. 2015). Specifically, cIMT is measured as the distance between these two parallel lines: lumen-intima and media-adventitia interfaces (Iana et al. 2015). It is important to highlight that the way in which the plaques are considered during the assessments could lead to different results in measurements. Despite the plaque-induced intima thickening, due to foam cells, smooth muscle cells, fibrous cap formation and macrophages, not all the studies using the cIMT technic include the assessment of the plaque in the measurements (Naqvi and Lee 2014). Increments in the value of the cIMT are related to a wide number of cardiovascular factors (Naqvi and Lee 2014; Iana et al. 2015). However, "normal cIMT" values are ranged according to age and sex. Aging seems to cause a steady increase in thickness in which men are more affected than women (Iana et al. 2015). Reference data gave by the American Society of Echography claim that cIMT values between the 25th to the 75th percentile are considered average, while values ≥ 75th percentile is classified as high and could increase the cardiovascular risk (Naqvi and Lee 2014; Iana et al. 2015).

2.2.2 Arterial Stiffness

Arterial stiffness is considered a reliable alteration marker for arterial structural and functional changes (Chen et al. 2017; Mozos et al. 2017), and it mainly occurs as a consequence of aging and/or arthrosclerosis process, as well as an indicator of cardiovascular risk (Chen et al. 2017; Mozos et al. 2017).

Arterial stiffness can be defined as the mechanical property of the artery to resistance to deformation (Shirwany and Zou 2010; Chen et al. 2017). This elasticity property is related with compliance and distensibility, but it is not interchangeable with them. Indeed, compliance and distensibility depend on the stiffness, as well as on arteries thickness and size (Shirwany and

Zou 2010; Chen et al. 2017). Considering that the inflammation plays a main role in atherosclerosis development, arterial stiffness affects primarily the large arteries (Mozos et al. 2017) and/or the larger central arterial system (i.e. the aortic system and its central branches) causing the mechanical fraying of lamellar elastin structures within the wall and the increase in the content of arterial collagen protein due to fibrosis together with the contemporary loss of arterial elastin (Shirwany and Zou 2010; Chen et al. 2017). As describe in the previous chapter (2.1 Regulation of vascular tone and blood flow in skeletal muscles), the arterial tree, with all the different types of arteries, allows to modulate the velocity of the pressure wave that is conducted from the heart to the larger vessels upstream (Mayer and Beretta 2009). This concept is known as "reflected wave", that is the interaction between the forward propagated wave, after the heart contraction, and the inherent impendence of the aorta that creates a relatively low velocity pressure wave (Mayer and Beretta 2009). The wave propagates distally encountering regions with different impendence, due to the different vascular wall and diameter of the arteries. Progressively, these mismatches not only amplify the forward propagating wave, but also produce a partial wave reflection (Mayer and Beretta 2009). The different reflected waves from many points of impedance mismatch are compounded to generate an aggregate, backward propagating wave (Mayer and Beretta 2009). Normally, in a healthy state, the backward wave arrives during the heart diastole, however, in a pathologic condition where arterial elasticity is reduced, the reflection wave is faster, arriving during the systolic phase of the cardiac cycle, causing an augment of the after load (i.e. the pressure needed to open the aortic valve) (Mayer and Beretta 2009).

Two are the most used techniques to assess the arterial stiffness: pulse wave analysis (PWA) and pulse wave velocity (PWV). For both measurements an applanation tonometry is generally

utilized (Mayer and Beretta 2009). PWA is a single measurement of the radial artery pressure waveform, which is then calibrated against the standard brachial artery blood pressure (i.e. systolic and diastolic point of the pressure curve). The augmentation index is calculated after mathematically transformed the pressure waveform into aortic waveform. The aortic wave form contains the first and second systolic peak, and the difference between them is the augmentation index, that normally is expressed as a percentage of the pulse pressure, and higher augmentation index values indicate a greater arterial stiffness (Mayer and Beretta 2009).

PWV is the velocity of the arterial pressure waveforms. It is calculated assessing simultaneously the pulse wave form in two different arteries (normally carotid and radial arteries, but could be also carotid and anterior tibial arteries) and measuring the distance between the two assessments point. The PWV is then calculated as the transient time between the two waves, grater values of PWV indicate a greater stiffness because of the quicker backward to the heart wave reflection (Mayer and Beretta 2009).

2.2.3 Glyceryl Trinitrate (GNT)

Glyceryl Trinitrate (GNT) is endothelium-independent vasodilator acting directly on the smooth muscle cells. The mechanism explaining its effects is still unclear. Some studies suggest GNT as a compound drug that, when de-nitrated, produces active NO (Vallance et al. 1989). However, there are also evidences proving that GNT can cause vasodilatation without increasing NO but activating sGC-cGMP pathway by lowering the cytosolic calcium (Mayer and Beretta 2009).

Veins dilatation is associated with a decreasing in left ventricular volume, therefore reducing the preload, and decreasing also the myocardial oxygen requirements. In the arterial tree, at the

arteriolar level, GNT induces relaxation, reducing the resistance (consequently reducing the afterload), and decreasing myocardial oxygen demand (Sidhu et al. 2015). GNT causes also coronary artery vasodilation improving the blood flow distribution in the myocardial (Sidhu et al. 2015).

GNT is commonly administrated sublingual as tablet or oral spray. The peak of the vasodilation usually occurs in 3-4 minutes (Ducharme et al. 1999).

2.2.4 Flow Mediated Dilatation (FMD)

Flow mediated dilatation (FMD) was first described by Celermajer et al in 1992 and, to date, it is the most common tool utilized to assess the endothelial function (Celermajer et al. 1992).

The use of the FMD test among the scientific community as non-invasive tools to measure endothelium (dys)function in the peripheral circulation, depends on the fact that its response resulted to be directly correlated with the endothelial-dependent vasodilator response to the injection of vasodilatory factors in the coronary arteries (Anderson et al. 1995; Khan et al. 2008). Indeed, FMD is generally measured on brachial, radial, popliteal, or superficial femoral arteries by a high-resolution Doppler ultrasound device in duplex modality.

A standard brachial-FMD maneuver consists in:

i) Baseline measurements: involving simultaneously assessments of vascular diameter images and blood velocity running into the vessel for two minutes;

ii) A brachial cuff pressure is placed around the elbow, immediately distal to the olecranon process, and was inflated in a supra-systolic pressure (>220 mmHg) to provide a stimulus for forearm ischemia for 5 minutes;

iii) The cuff is rapidly deflated and two minutes of simultaneously assessments of vascular diameter images and blood velocity is taken to quantify the reactive hyperemia occurring as well as the changes in vessel diameter.

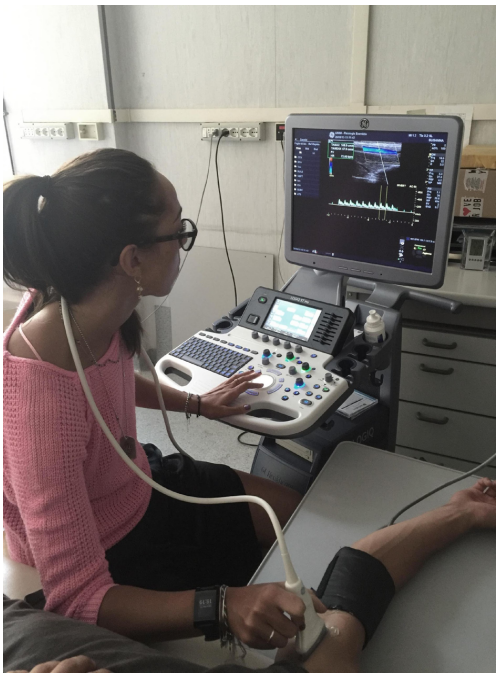

Figure 6. Example of Flow Mediated Dilatation Assessment from our Lab: Human Exercise Physiology Lab, Department of Biomedical Sciences for Health, Università degli Studi di Milano.

The FMD is then quantify as the maximal change in artery diameter after cuff release, expressed as a percentage increase (%Δ) above baseline (Harris et al. 2010):

(Peak – baseline diameter) / baseline diameter · 100.

Brachial artery blood flow was estimated by using the analyzed software data of mean blood velocity (v_{mean}) and arterial diameter as:

$$\text{Blood flow (ml} \cdot \text{min}^{-1}) = v_{mean} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60.$$

It should be clarified that the hyperemic FMD response is causes by the tissue ischemia and dilatation of downstream cuff vessels, and therefore a hyperemic response during the cuff release occurs in order to fill the dilated resistance vessels downstream the cuff (Corretti et al. 2002).

Indeed, maximum vasodilation upstream the cuff occlusion occurs subsequently to the reactive hyperemia (usually, in young heathy human around 20 seconds after the cuff release) (Figure 7), which produces an increase in shear rate, thus stimulating endothelial cells to release vasoactive factors (i.e. FMD is commonly considered mainly NO-dependent) (Pyke and Tschakovsky 2005).

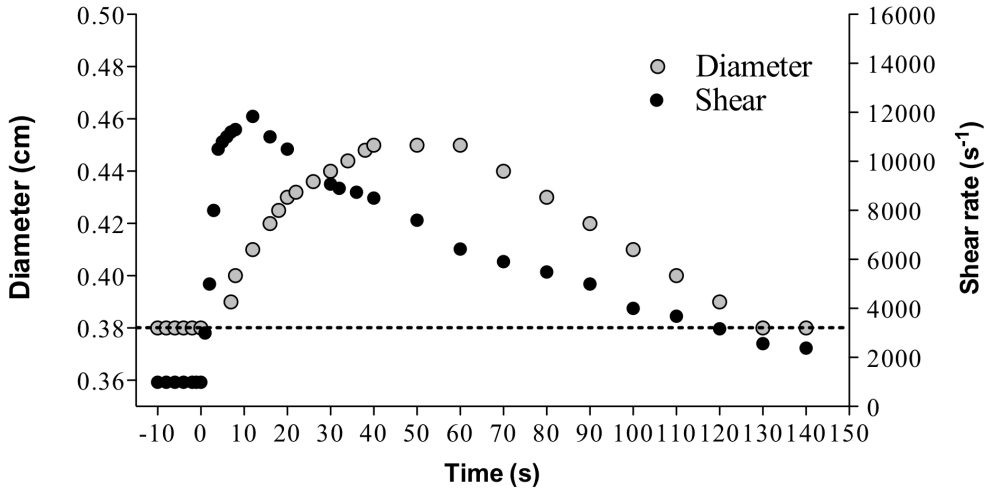

Figure 7 On the model propose by Wu et al., 2015 on *The use of shear rate-diameter dose-response curves as an alternative to the flow-mediated dilation test:* an example di FMD (%) and shear rate (s^{-1}) response trend over the cuff release period (corresponding to 0 time in Figure).

As previously mention, the shear rate is the laminar shear force running in parallel to vessels long axis that is transduced by the luminal mechanoreceptors of endothelial cells (Niebauer and Cooke 1996). Being the shear rate one of the main factor causing the FMD response, a quantification of reactive hyperemia, in terms of shear rate, is commonly given to assess the amount of stimulus on the endothelial cells (Pyke and Tschakovsky 2005):

$$\text{SR } (s^{-1}) = 8 \cdot v_{mean} / \text{vessel diameter}$$

Moreover, some authors underline the importance of normalizing the percentage of vasodilatation by the shear rate stimulus necessary to obtain the peak of vasodilatation in order to eliminate the influences of variable shear rate and provide a meaningful FMD (Pyke and Tschakovsky 2005; Padilla et al. 2008).

In 2013 Wray et al., questioned if the FMD test was a good indicator of the NO-bioassay, considering the literature ambiguities, and the progressively widespread clinical use of the FMD (Wray et al. 2013). The results have shown that despite the use of an endothelial NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), the FMD response was just blunted, and that the difference disappeared when the FMD was normalize by the shear rate (Wray et al. 2013). These findings challenge the assertion of FMD as a noninvasive tool to assess the endothelium NO-derived vasodilation in young healthy humans, and call into question the necessity of new methods to realize that purpose.

Lastly, in view of the above, it is important to recognize that occlusion duration, cuff placement, position of the probe and pre-condition of the subject (i.e. sleep deprivation, caffeine assumption, phase of the menstrual cycle, antioxidant supplementation etc.) could affected the FMD and the role of NO in the hyperemic response (Hashimoto et al. 1995; Mullen et al. 2001;

Corretti et al. 2002; Takase et al. 2004; Harris et al. 2010). Accordingly, it is important to control all these factors (Hashimoto et al. 1995; Mullen et al. 2001; Corretti et al. 2002; Takase et al. 2004; Harris et al. 2010).

2.2.5 Passive Limb Movement (PLM)

The passive limb movement (PLM) is a relatively new non-invasive approach to evaluate the NO-dependent endothelial function (Trinity et al. 2012). PLM maneuver generates an hyperemic response in the peripheral as well as in the central hemodynamic without the presence of any increase in metabolism associated with active exercise (Trinity et al. 2012).

The PLM methodological advantage in comparison to FMD is that PLM is not relying on the measurement of small changes in vessel diameter (Venturelli et al. 2017). Indeed, PLM protocols is performed on the common femoral artery of the passively moved leg, distal to the inguinal ligament and proximal to the deep and superficial femoral bifurcation with the use of an ultrasound Doppler system (Trinity et al. 2012, 2015; Venturelli et al. 2017). Following the PLM guide lines (Trinity et al. 2012, 2015; Venturelli et al. 2017) the test consists in:

i) Let the subjects rest for at least 20 minutes in an upright-seated position (the same position will be maintain throughout all the test);

ii) Collect 60 seconds of resting and baseline central and peripheral hemodynamic data;

iii) An external operator applies 60 s of passive knee extension and flexion while the same measures are recorded. The operator moves the subject's lower leg through a 90° range of motion (180–90° knee joint angle) at 1 Hz. Every knee flexion and extension take 1 second, and after 1 minute of movement, the leg is maintained fully extended;

iv) Collect 120 seconds post-movement data.

Figure 8. Example of Passive Limb Movement from our Lab: Human Exercise Physiology Lab, Department of Biomedical Sciences for Health, Università degli Studi di Milano.

Ultrasound images of the femoral artery and its relative blood flow velocity are constantly recorded and utilized then to calculate the blood flow as:

$$\text{Leg Blood Flow} = \text{blood flow velocity}_{\text{mean}} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60$$

However, one minute of PLM seems to not only increase the peripheral blood flow but also induces a cardio-acceleration increasing the cardiac output due to an increase in the heart rate (McDaniel et al. 2010a) explained by an increase in the feedback afferents (type III afferents fibers) from skeletal muscle (Nóbrega and Araújo 1993; McDaniel et al. 2010a). Thus, to minimize the impact of PLM on central hemodynamic, a modified PLM approach is also utilized. Single PLM (sPLM), instead of move the leg for 60 seconds, consists in one passive

knee flexion and extension, which took 1 s, after which the leg was maintained fully extended for the remaining 59 seconds (Venturelli et al. 2017). sPLM generates a significant peripheral hyperemic response with a minimum activation of afferents feedback minimizing the increase in the central hemodynamics due to the cardio-acceleration (Venturelli et al. 2017).

The PLM efficacy in assessing the endothelial NO-dependent were discussed by Trinity et al. (2015). In this study the effects of aging on the PLM response, therefore in the NO-bioavailability, was assessed (Trinity et al. 2015). They found that the PLM response in young healthy men causes a large increase in the leg blood flow and this hyperemic response is primarily mediated by NO.

However, leg blood flow response in old people was significantly reduced compare to the young. Moreover, when they tried to attenuate NO influence (by an NOS inhibition, L-NMMA) on the PLM response, the impact on the peak and the overall hyperemic response in the old was further reduced (Trinity et al. 2015), underling the big NO-dependency in the PLM response (Trinity et al. 2015).

Chapter 3- Role of physical activity to promote vascular health: influences of different types of exercise on vascular function and structure.

Regular physical exercise is the most important non- pharmacological means to promote, maintain or improve health. The main roles of regular physical activity are: i) to prevent cardiovascular diseases due to the effects of exercise intervention on blood pressure, lipids profile, insulin resistance and obesity (Thompson et al. 2003); ii) improve physiological functions (Carol Ewing Garber; Bryan Blissmer; Michael R. Deschenes; Barry A. Franklin; Michael J. Lamonte; I-Min Lee; David C. Nieman; David P. Swain 2011); iii) have some benefits also in chronic diseases with a vascular etiology such as diabetes and dementia (Barnes 2015).

A meta-epidemiological study, investigating the comparative effectiveness of exercise and drug interventions on mortality outcomes, reveals that exercise and medications have potentially the same effects in terms of benefits on mortality, suggesting that exercise intervention could be used as a valid alternative, or alongside the normal drug therapy (Naci and Ioannidis 2013). Additionally, Anderson et al, (2016) shown that exercise- based cardiac rehabilitation reduces hospitalization and improves the related quality of life, proposing the exercise as an active rehabilitative component (Anderson and Taylor 2014).

Recently, Daniel J. Green and Kurt J. Smith stated that: "*exercise is a vascular medicine*" (Green et al. 2017b). Indeed, regular physical exercise not only reduces or prevents the above mentioned cardiovascular risk factors (Thompson et al. 2003; Joyner and Green 2009; Carol Ewing Garber; Bryan Blissmer; Michael R. Deschenes; Barry A. Franklin; Michael J. Lamonte; I-Min Lee; David C. Nieman; David P. Swain 2011; Barnes 2015), hence improving vascular

functionality , but it seems to have a direct effect on the arteries (Green et al. 2008, 2017a) inducing functional and structural arterial adaptations (Green et al. 2017b). In addition, these improvements in the overall arterial health may be primarily related to enhancements in endothelium health and function.

However, data demonstrating that physical exercise directly influences vascular functionality are controversial, and primarily related to assessments with FMD rather than PLM technic. Specifically referring to FMD data, different types of exercise stimulus (i.e. single acute bouts *vs* long term training) might trigger different patterns in vasculature response.

Regarding the PLM, it was just recently introduced as a new approach to assess vascular function (such as NO endothelial-dependent functionality) (Trinity et al. 2012), therefore not utilized to investigate the role of the exercise yet. PLM causes the movement-induced hyperemia in the lower leg by removing the increase in metabolism associated, and highlighted that the muscle pump (Wray et al. 2005), perfusion pressure (Trinity et al. 2011), and afferent feedback (Venturelli et al. 2012) not only they have a substantial impact on the PLM hyperemic response, but also they could change after either an acute and/or long term exercise training, leading to the idea that different PLM responses might be expected after a single bout of exercise or a training protocol. However, how the different types of exercise stimulus affect the PLM response is still unknown.

In 2013 Dawson et al., introduce the idea of the "*exercise paradox*", which represents a transient period of increase in cardiovascular risk occurring after a single bout of exercise based on the reduction in FMD values (Dawson et al. 2013). Indeed, as shown in Figure 9, after a single bout of exercise the FMD response presents a biphasic pattern with an immediate decline, namely *nadir* and occurring soon after exercise termination, which is then followed by a restoration, or

often super-compensation, of the response (Dawson et al. 2013). Regarding the physiological factors that could affect the magnitude, direction and nature of the biphasic response can be reported: oxidative stress (Finaud et al. 2006), shear rate stimulus (Thijssen et al. 2009b, 2011b, Tinken et al. 2009, 2010), baseline arterial diameter (Celermajer et al. 1992; Corretti et al. 2002; Pyke and Tschakovsky 2005) and sympathetic nerve activity (Atkinson et al. 2015). In the other hand, there are also some differences in FMD values in response to different methodological approaches and types of exercise adopted. Concerning the methodological approaches and the different types of exercise it seems that the main factors influencing the magnitude of the biphasic FMD response are related to exercise protocol (e.g. the type of exercises and intensity), participants fitness level and time in which the FMD is measured from exercise cessation (e.g., immediately after and/or hours from exercise cessation).

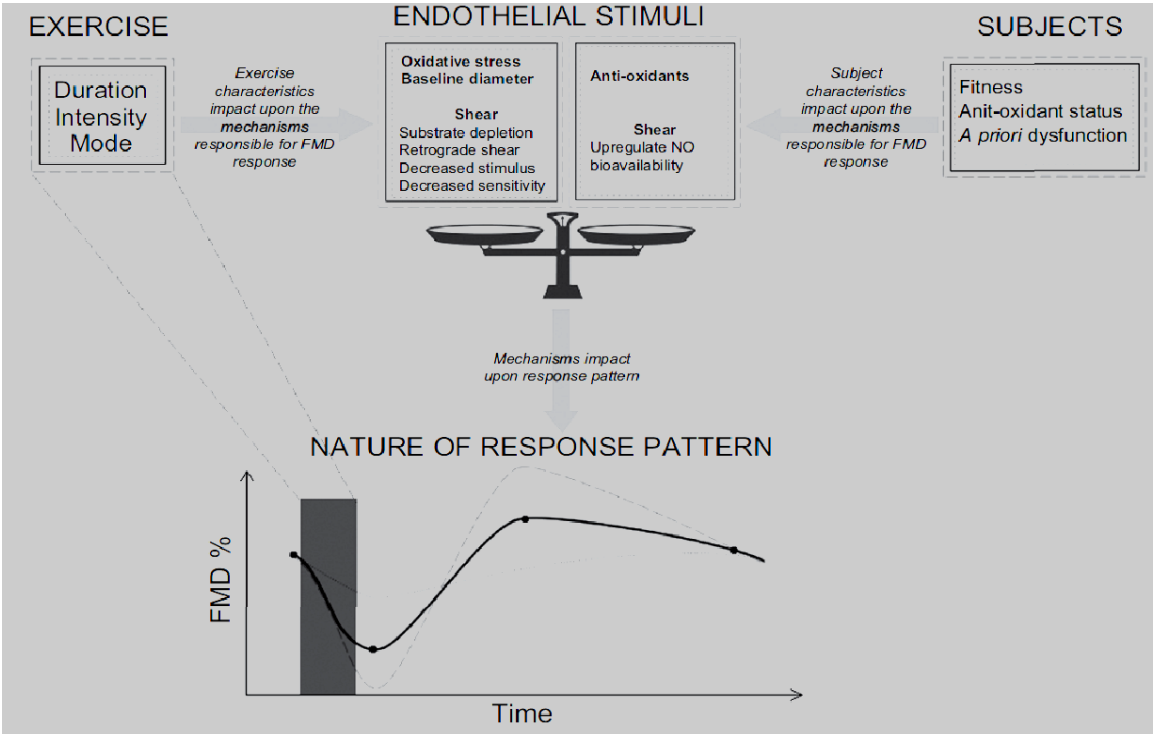

Figure 9. Biphasic response in flow-mediated dilatation (FMD) after an acute bout of exercise., from Dawson EA et al., *Effects of acute exercise on flow-mediated dilatation in healthy humans*, (J Appl Physiol, 2013).

In detail, in a heathy population, a whole body single bout of exercise performed with an intensity ranging from moderate to high, it seems to induce a transitory reduction in the FMD (Rognmo et al. 2008; Katayama et al. 2013; Birk et al. 2013; Atkinson et al. 2015). However, also the exercise duration plays a role, highlighting no changes in FMD with the same exercise intensity but shorter time of exercise duration (McClean et al. 2015). On the contrary, when a pathological population is considered, no changes (Currie et al. 2012) or increases (Tjønna et al. 2011) in the FMD values are detected after a moderate or high- intensity single bout of exercise.

Moreover, when exercises involve small muscle mass (e.g. handgrip exercise), and they are proximally to the artery's site investigation (e.g. brachial artery FMD), a reduction in the FMD is detectable at lower intensities and durations compare to whole body exercise (Gonzales et al. 2011).

Additionally, trained individuals reported a larger baseline vessel diameter that may likely influence also the maximum blood flow via a greater reactive hyperemia after the cuff release (Rognmo et al. 2008). Indeed, regardless the intensity of the exercise (moderate or high), the FMD in healthy aerobic-trained individual is higher after an acute dose of exercise (Johnson et al. 2012), suggesting that the level of fitness may play a crucial role in endothelial cells-response to shear stimulus due to a vascular remodeling (Pyke and Tschakovsky 2005; Rognmo et al. 2008).

Under a physiological point of view, the *FMD nadir* can be due to an increase in exercise-induced oxidative stress, together with an elevated shear rate (occurring during exercise and due to the increase of the peripheral blood flow according to the muscles oxygen request) that likely lead to a transitory depletion of extra-cellular L-arginine. Consequently, it may limit NO re-

synthesis and generate an inability to dilate vessels in response to a given FMD-shear stimulus immediately after a single bout of exercise (Dawson et al. 2013). Also, the baseline artery diameters' size can affect post-exercise FMD. Larger arteries have a diminished dilator capacity than smaller ones (Celermajer et al. 1992; Pyke and Tschakovsky 2005; Thijssen et al. 2008). Post-exercise baseline diameter is larger due, as above mentioned, to the increase in shear rate occurring in conduit arteries causing vasodilation (Gonzales et al. 2011; Birk et al. 2013; McClean et al. 2015) and determines a decrease in endothelial cells response to a shear stimulus during FMD maneuver performed after exercise. Another mechanism proposed to explain the reduction in post-exercise FMD could be ascribed to the sympathetic nervous activity (SNA). Indeed, immediately after an exercise bout, FMD is affected by SNA interfering on the NO–dilator pathway during FMD maneuver (Atkinson et al. 2015), and generating an increase in retrograde shear rate during exercise that in turn affects FMD (Birk et al. 2013).

Literature about the long-term effects of exercise training on vascular functionality seems to agree in affirming that endothelial functionality, and consequently the FMD values, are improved after an exercise training protocol. However, the magnitude of the improvement could depend on the type of population recruited (healthy individuals *vs* pathological populations), training duration and modality (i.e. aerobic, resistance, combined or handgrip training).

In detail, metabolic and cardiovascular diseases patients present a poor vascular functionality worsening over the years. Thus, in these patients baseline vascular health status is reduced compared to matched heathy control and partially explaining their greater enhancement in FMD values after a training (Green et al. 2017b).

To explain the physiological effects of a long-term exercise on vascular functionality a schematic resume is propose in Figure 10.

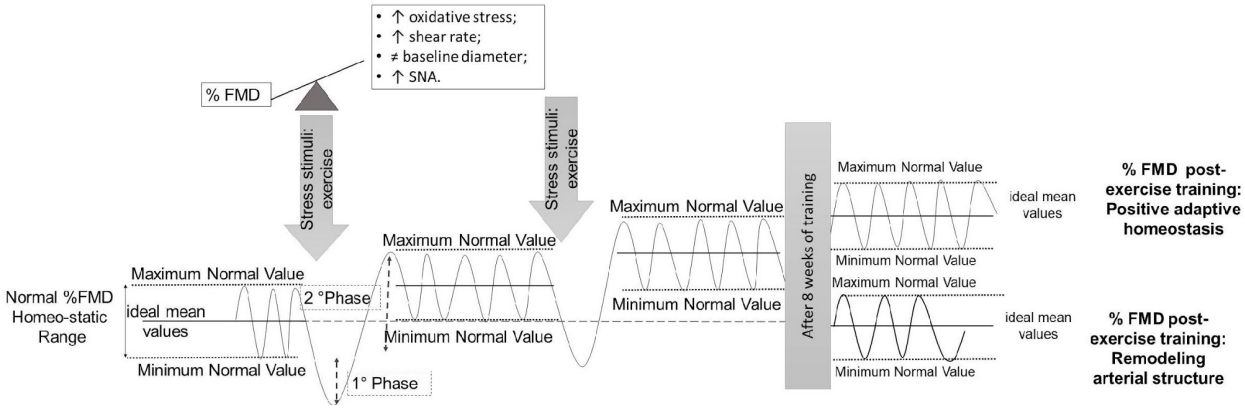

Figure 10. Schematic overview of physiological adaptations in FMD (%) after long-term exercise training.

As above stated, after a single bout of exercise the homeostatic FMD range is altered, causing a decrease in the first phase of the biphasic response model (Dawson et al. 2013) as shown in Figure 9 and 10. However, the endothelial function can be then restored or upregulated according to the stress stimulus received (Davies 2016b, a) during the second phase of the biphasic model of FMD (Figure 9-10) (Dawson et al. 2013).

Significant improvements in FMD, as a consequence of a long-term training protocols, are likely mediated by the repetition of the FMD biphasic model throughout the exercise training program allowing the enhancement of the "normal homeostatic range" (Figure 10). The most important physiological pattern is shear rate, which appears to have a crucial role in triggering the positive adaptation process (Tinken et al. 2010; Birk et al. 2012; Green et al. 2017b, a).

The first observation suggesting that hemodynamic forces, such as the mechanical forces related with shear stress and/or blood pressure, are important in adaptation of the vasculature was at the end of the 19th century (Thoma R 1893). Indeed, Thoma observed that the development of

branches in the blood vessels was strictly related to the blood flow velocity, where no many branches were developed in those blood vessels when blood velocity was slower (Thoma R 1893). Later in 1975, Rodbard proposed the first model of prediction of flow-dependent, endothelium-mediated dilation and remodeling (Rodbard 1975) (Figure 11). Rodbard described four main steps resuming the positive adaptations on vascular functionality (Figure 11) (Rodbard 1975):

- Step 1: set point, the endothelium is exposed to a normal viscous drag force or shear rate;
- Step 2: the endothelium is exposed to repetitive increments in blood flow (i.e. increment in blood flow occurring every single bouts of a training exercise protocol);
- Step 3: the endothelium reacts with functional change such as vasodilatation that tends to homeostatically modify the initial increase in drag force or shear;
- Step 4: vessel remodeling could occur whereby drag forces are “structurally” normalized and in response to prolonged or repetitive periods of exposure to change in flow and shear.

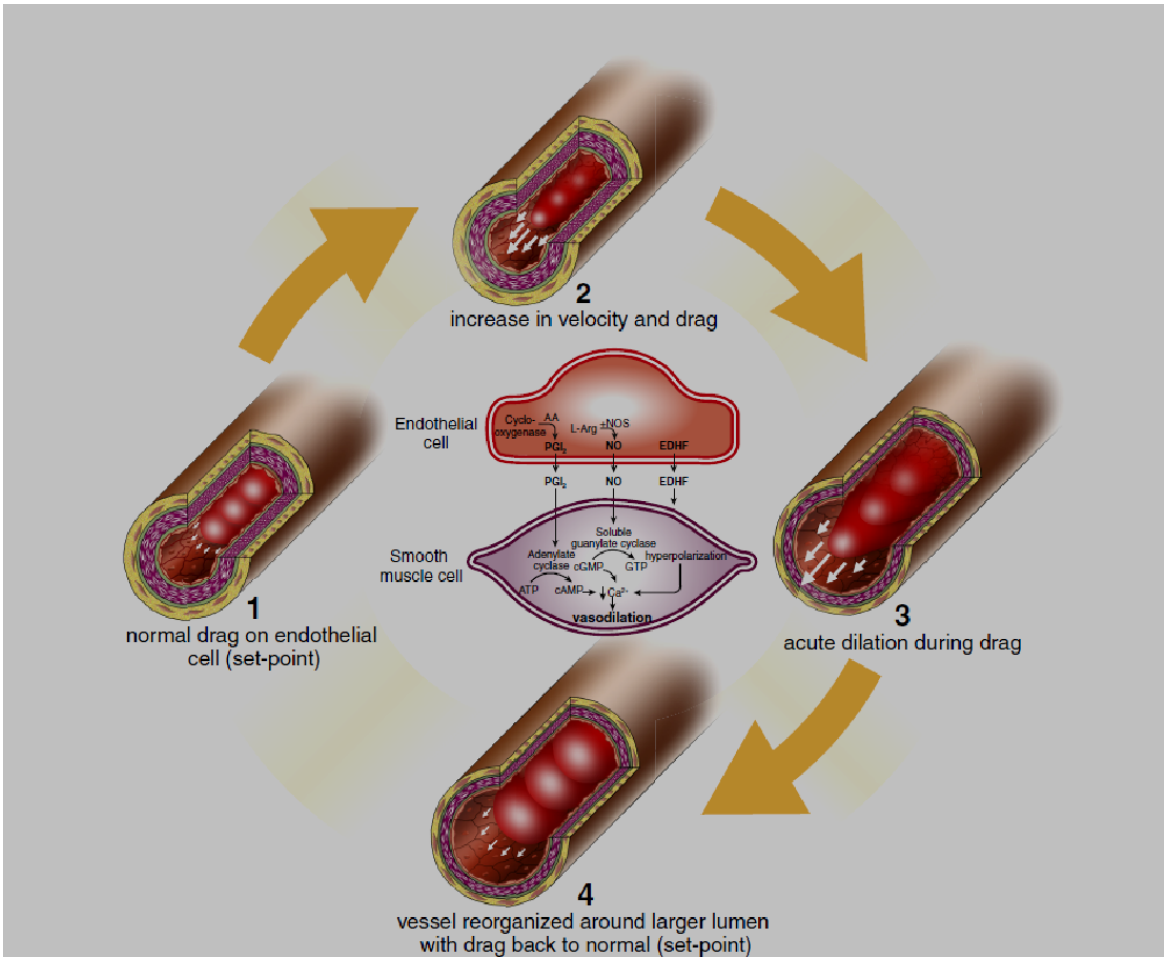

Figure 11. Rendering of "flow-dependent, endothelium-mediated dilation" and remodeling based on Rodbard's prediction, from Green JD et al., *Vascular adaptation to exercise in human: role of hemodynamic stimuli*, (Physiol Rev, 2017).

To date, not only the Rodbard's assumptions were verified in vivo in animals and humans, but it is also out of doubt that the positive vascular adaptations are strictly dependent on the endothelium reaction.

Indeed, the endothelium functionality was tested in vivo in a dogs study in which the progressive infusion of acetylcholine, leading to an increase in the blood flow, remarkably increased the arterial diameter, while the dilatory response was abolished after the mechanical removal of the

endothelial cells lay (Pohl et al. 1986). Moreover, it was also shown that the exercise-vasodilation in canine epicardial coronary artery turned in vasoconstriction after mechanical endothelial denudation (Berdeaux et al. 1994).

In humans the effects of the repetitive increments in shear rate throughout an exercise training protocol were investigated on the brachial artery by Tinken et al. (2010) and Birk et al., (2012). Specifically, Tinken et al., tested, by FMD determination, the vascular functionality in response to an 8-week bilateral handgrip training. During every single session of training shear-stress responses were attenuated by partially occluding just one of the two limbs involved in the bilateral handgrip training with a blood pressure cuff. After the 8 weeks, FMD percentage increased only in the un-cuffed arm, whereas no changes were evident in the cuffed arm, revealing that exercise-induced changes in shear provide the principal physiological stimulus to adaptation in flow-mediated endothelial (Tinken et al. 2010).

Birk and colleagues adopted a similar approach to this scientific problem. Indeed, using a 8-weeks cycle training, they assessed the FMD in both cuffed and un-cuffed brachial arteries pre- and post-training (Birk et al. 2012). After training, adaptations in flow-mediated endothelial function occurred in the non-exercising upper limbs, specifically only in the un-cuffed arm, underlying that: i) the role of cycling impact on brachial artery blood flow and shear stress; ii) vascular adaptations occurred only in the arm completely exposed to the shear stimuli (Birk et al. 2012).

There are also evidences revealing that long-term exercise training is associated with enlargement of the baseline arterial diameter in animal model (Green et al. 2017a), suggesting that an arterial structure remodeling occurred (Green et al. 2017b, a). Based on these animal studies, it was proposed that initial functional vascular readjustments, in terms of greater

vasodilation, to normalize the shear stimulus become permanent, such as bigger baseline diameter, where the stimulus is continuously repeated. As a consequence of this arterial structure remodeling the initial functional vascular readjustments return towards baseline values (Laughlin et al. 2012).

Similar results were also found in humans. Indeed, studies with long-terms exercise training revealed a progressive increase in the brachial artery dilatory response to an ischemic exercise stimulus, that is a surrogate measure for conduit artery structure (Naylor et al. 2005), whereas no changes were detected in baseline diameter (Tinken et al. 2009, 2010; Birk et al. 2012; Weber et al. 2013).

All together these results confirm the hypothesis that improvement in vasodilator function (i.e. FMD), throughout an exercise training program, were replaced by structural remodeling (i.e. baseline diameter and/or brachial artery vasodilator response to an ischemic exercise stimulus) returning the vasodilator function toward baseline values.

Chapter 4 – Study 1

Respiratory muscle training positively affects vasomotor response in young healthy women

4.1 Materials and methods

4.1.1 Participants

Twenty-four young recreationally active female participants (table 1), volunteered for the study and were randomly assigned to two group: respiratory muscles training group (RMT, n = 12) and sham group (SHAM, n = 12). The inclusion criteria were: (i) regular menstrual cycles (26 to 35 days) for at least 3 months prior to start the study, (ii) being clinically healthy, (iii) free from cardiovascular diseases, (iv) free from medications, including hormonal contraceptives and oral supplements, and (v) normal lung function as determined by medical screening and functional assessments. Each participant signed an informed consent after being fully informed on the purpose of the study and experimental procedures. The Institutional Review Board approved the study, which was performed in accordance with the principles of the 1964 Declaration of Helsinki.

Table 1 Participants' characteristics.

	RMT	SHAM	*P* value
Participants (n.)	12	12	-
Age (years)	25 ± 8	29 ± 9	0.27
Stature (m)	1.67 ± 6.01	1.66 ± 8.00	0.70
Body mass (kg)	64.6 ± 10.4	61.5 ± 9.9	0.47
Body mass index (kg/m^2)	21 ± 3	19 ± 3	0.47

RMT, Respiratory muscle training. Data are expressed as mean ± SD.

4.1.2 Study design

Before and after RMT, participants were tested at about the same time of the day, and at the same day of the menstrual cycle (early luteal phase) in a climate-controlled laboratory (constant temperature of 20 ± 1 °C and relative humidity of 50 ± 5 %). Participants documented their menstrual cycle in a personal diary throughout the study, which was used to assess the early luteal period. Subjects fasted overnight, abstained from caffeine and other similar substances for 12 hours, and did not participate in heavy exercise for 48 hours. Post interventional experiments were performed 24 to 48 hours after the last session to prevent examination of its acute effects. Moreover, an extra visit was designed for a subgroup of eight participants, who were randomly selected, in order to quantify the possible increase in brachial artery blood flow and in other central parameters, such as blood pressure, cardiac output and

heart rate during a single session of respiratory muscle exercise. These data were collected after the pre-screening tests and before starting the RMT program.

4.1.3 Measurements

Heart rate variability (HRV)

HRV analysis was used to determine the balance between the sympathetic and parasympathetic nervous system (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996; Chen et al. 2015). Subjects laid supine for approximately 10 minutes after which the electrocardiogram (ECG) signal was recorded for other 10 minutes. ECG signal was recorded by BIOPAC MP150 data acquisition (BIOPAC, ECG100C Santa Barbara, CA USA) and AcqKnowledge software allowed using multiple applications. Subsequently, data were exported and analyzed with Kubios HRV software (ver. 2.2, available on http://kubios.uef.fi.) that selected the clearest 5 min waves trace of the entire recording ECG signal was analyzed in the frequency-domain, assessing the frequency band between 0.01 and 0.4 Hz. Low Frequency (LF, between 0.04 and 0.15 Hz) represented the sympathetic nervous drive activity and baroreceptor control (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. 1996), while High Frequency (HF, between 0.15 and 0.4 Hz) represented the vagal activity. Given that the two branches of the autonomic nervous system operate in a coordinated manner, changes in the amount of HF and LF can provide a numerical index of the direction and magnitude of reciprocal alteration in sympatho-vagal balance (Milicević and Mili~evi} 2005). Therefore, the LF/HF ratio was also calculated as an index of the sympatho-vagal balance.

Flow-mediated dilation (FMD)

FMD measurements of the brachial artery were performed according to the recommended procedures (Harris et al. 2010). Before FMD, the subjects laid supine for approximately 20 minutes, and then a blood pressure cuff was placed on the forearm immediately distal to the olecranon process to provide a stimulus for forearm ischemia. A 12- to 14-MHz linear array transducer, attached to a high-resolution ultrasound machine (Vivid I, GE Medical Systems, Milwaukee, WI, USA) was used to image the brachial artery in the distal third of the upper arm. When an optimal image was obtained, the probe was held stable and longitudinal B-mode images of the lumen-arterial wall interface were acquired. Continuous Doppler velocity assessments were also obtained using the ultrasound and were collected using the lowest possible insonation angle (always <60°). Following baseline assessments, a forearm blood pressure cuff was inflated to 250 mmHg for 5 min. Diameter and flow recordings resumed 30 s prior to cuff deflation and continued for 2 min post-deflation in accordance with recent technical specification (Corretti et al. 2002; Harris et al. 2010; Wray et al. 2013). The FMD data were exported in AVI format and analyzed using commercially available software (Brachial Artery Analyzer for Research, Medical Imaging Applications, LLC, Coralville, IA), which is largely independent of investigator bias. Furthermore, these data were verified by an expert operator blinded towards the experimental condition (RMT *vs* SHAM and pre *vs* post).

FMD was quantified as the maximal change in brachial artery diameter after cuff release, expressed as a percentage increase (%Δ) above baseline:

(Peak – baseline diameter) / baseline diameter · 100.

Brachial artery blood flow was estimated by using the analyzed software data of mean blood velocity (v_{mean}) and arterial diameter as:

$$\text{Blood flow (ml} \cdot \text{min}^{-1}) = v_{mean} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60.$$

The shear rate (SR) was calculated post cuff release using the following equation:

$$\text{SR } (s^{-1}) = 8 \cdot v_{mean} / \text{vessel diameter}$$

The cumulative SR, corresponding to the reactive hyperemia post cuff release (total SR from cuff release to time to peak), was integrated (area under the curve, AUC) by using the trapezoidal rule, and calculated as:

$$\Sigma[yi \cdot (x \cdot (i\text{-}i) - xi) + (1/2) \cdot (y \cdot (i\text{-}i) - yi)\ (x \cdot (i\text{-}i) - xi)]$$

The cumulative/integrated SR (AUC) reflects the amount of mechanical stimulus applied on the endothelium during the cuff release hyperemic response at time to peak. Considering that FMD is primarily dependent on the endothelium response to mechanical stimuli, the %FMD was therefore divided by cumulative SR (%FMD/SR) (Pyke and Tschakovsky 2005).

Pulmonary function

Before and after RMT, participants underwent a pulmonary function evaluation via spirometry dynamic lung volumes (forced vital capacity, FVC; forced expired volume at 1st second, FEV_1; and maximal voluntary ventilation, MVV) were obtained. Residual volume (RV) was also determined using a nitrogen washout method (Pyke and Tschakovsky 2005) and total lung capacity (TLC) was calculated as the sum of RV+ FVC. Maximal inspiratory pressure (MIP) was measured at the mouth using a portable manometer equipped with a mouthpiece (S&M Instrument Company Inc., mod. PortaResp, Doylestown, PA). After familiarization with the

manometer, participants were asked to inspire as deep as possible following a normal expiration. They repeated the maneuver three times and the highest value was considered for comparisons before and after RMT.

Respiratory muscle training (RMT)

Before the first session of RMT, a sub-group of participants (n=8) reported to the laboratory for an additional session of respiratory muscle exercise to assess the acute effects of exercise on brachial artery blood flow, SR and central parameters such as heart rate, stroke volume and mean arterial pressure. During this session, continuous Doppler brachial blood velocity and vessel diameter were assessed by ultrasound. Heart rate (bpm) and mean arterial pressure (MAP, mmHg) were determined by electrocardiography and by finger photoplethysmography (Finometer PRO, Finapres Medical System, Amsterdam, The Netherlands) positioned at the heart level, respectively. Stroke volume (SV, ml) was automatically calculated using the Modelflow method (Finapres Medical System), with cardiac output (CO, $l \cdot min^{-1}$) calculated as the product of SV and heart rate. The additional session consisted of two bouts of one-minute of respiratory muscle exercise performed at two different intensities: the volume was set at 50% of FVC for both intensities while the frequency was set at 20 and 30 breaths per minute during the first and second bout of exercise, respectively. A 3-minute recovery period was given between the two bouts. Before any exercise, a baseline measurement of 30 seconds was recorded. The brachial blood velocity and brachial artery diameter assessments were then used to calculated blood flow ($ml \cdot min^{-1}$) and SR (s^{-1}). Brachial vascular conductance (BVC) was also calculated as the ratio between blood flow and MAP:

BVC (ml· min^{-1}· mmHg^{-1}) = brachial blood flow / MAP. All the parameters were average over a time window of 30 seconds during baseline and the last 10 seconds of each recording.

RMT was performed for 8 weeks, 3 sessions per week for 15–30 min each, which included a warm-up and cool-down using a SpiroTiger® device (SpiroTiger® Medical, Idiag AG, Fehral- torf, Switzerland) allowing for isocapnic hyperpnoea (Figure 12). During the first week, participants were familiarized with the instrumentation and did specific respiratory muscle stretching. Starting values of the volume and frequency of respiratory cycles were determined as a percentage of FVC and MVV. In particular, the initial volume was set at 50% FVC, while initial breath frequency (f_b) was calculated as follows (Spengler et al. 1999; Markov et al. 2001):

$$f_b = MV / (1.3 \cdot 0.5 \cdot FVC)$$

where respiratory minute volume (MV) was set at 60% of MVV.

Fig 12. A schematic drawing of the study design.

The workload was increased by ~ 15% every week by alternating either volume or f_b. If necessary, small adjustments were applied according to participants' adaptability. The RMT was monitored by an expert operator. The properties of the training device allowed personalized respiratory training through maximal inspirations and expirations. To avoid hypocapnia due to hyperventilation, the device features a two-way piston valve connected to a rebreathing bag. As the subject breathed out through the mouthpiece, the rebreathing bag stored part of the expired air, which contained increasing concentrations of CO_2. Once the rebreathing bag was filled to its capacity, a valve opened and allowed the rest of the expired air to be released into the environment. The valve shut when expiration finished and inspiration began. Inspiration emptied the rebreathing bag first (containing increased concentrations of CO_2), then the valve opened and some fresh air from outside was inspired at the end of each inspiration. This apparatus allows for respiratory cycles with high frequency while maintaining isocapnic hyperpnoea. The familiarization week represented the placebo-training paradigm of SHAM for the following seven weeks, for a total of eight weeks of SHAM intervention.

Statistical analysis

Statistical analysis was performed using a statistical software package (IBM SPSS Statistics v. 22, Armonk, NY, USA). Shapiro-Wilk test was used to check the normal distribution of the sampling. A sample size of 12 healthy women was selected to ensure a statistical power higher than 0.80 based on a preliminary study on the difference between the two groups in SR. A two-way, mixed model Intraclass Correlation Coefficient (ICC) and the Standard Error of Measurements calculation as a percentage (SEM%) were utilized to assess inter-session reliability. ICC values were considered very high if >0.90, high if between 0.70 and 0.89, and

moderate if between 0.50 and 0.69. The sensitivity in detecting the differences between the two trial sessions was checked by calculating the minimum detectable change at 95% confidence as a percentage (MDC95%) (Donoghue et al. 2009). %FMD was normalized by cumulative SR (%FMD/SR). For this reason, the mean percentage difference with lower and upper CI was calculated also for RMT-induced changes in cumulative SR (AUC).

To assess significant effects of RMT on pulmonary function, HRV, and FMD parameters, a paired Student's t-test was applied within each group, while a Student's t-test was used to assess the differences between groups. Two separate two-way ANOVAs for repeated measures (training x time) were applied to disclose possible differences in the blood flow kinetics post cuff release during FMD. A two-way ANOVA (group x time) was utilized to determine possible differences between groups in the blood flow kinetics post cuff release during FMD. When appropriate, the post hoc Holm-Sidak test was utilized for the location of the difference. Statistical significance was accepted at $P<0.05$. Unless otherwise stated, values are expressed as mean ± standard error (SE).

4.2 Results

At baseline, there were no significant differences in subject demographics between RMT and SHAM groups (Table 1).

During the two single bouts of RMT (n = 8) central parameters and peripheral blood circulation changed as follow: while MAP and CO did not change within the first intensity (101 ± 3 to 108 ± 5 mmHg (+7 %; P = 0.11) and 3.49 ± 0.38 to 3.68 ± 0.55 $l \cdot min^{-1}$ (+6%; P = 0.059)), both parameters increased throughout the second intensity (101 ± 2 to 114 ± 2 mmHg (+13 %;

P<0.05) and 3.40 ± 0.52 to 4.28 ± 0.43 l·min^{-1} (+26%; P<0.05)). Heart rate rose from 70 ± 5 to 83 ± 7 bpm (+18%; P<0.05) and from 74 ± 2 to 114 ± 2 bpm (+26%; P< 0.05), respectively. Blood flow increased within the first and the second RMT intensities, respectively (47.8 ± 0.9 to 116.9 ± 2.2 ml · min^{-1} (+145%; P<0.001) and from 50.9 ± 1.6 to 173 ± 3.37 ml·min^{-1} (+241%; P<0.001)). Also, BVC increased significantly throughout the two trials (0.47 ± 0.19 to 1.08 ± 0.12 ml·min^{-1}·mmHg $^{-1}$ (+129 %; P<0.05) and 0.50 ± 0.19 to 1.53 ± 0.17 (+203 %; P<0.05) ml·min^{-1}·mmHg $^{-1}$). SR increased from 211 ± 3 to 393 ± 5 s^{-1} (+87%; P<0.001) and from 216 ± 3 to 466 ± 8 s^{-1} (+115%; P<0.001) during the first and second RMT exercise intensities, respectively. Brachial artery diameter enlarged from 0.326 ± 0.001 to 0.377 ± 0.003 cm (+15%; P<0.001) and from 0.333 ± 0.001 to 0.404 ± 0.001 cm (+21%; P<0.001) during the first and second RMT exercise bout, respectively.

Reliability and sensitivity of the measurements

ICC and SEM% for the investigated variables are reported in Table 2. Inter-session reliability for respiratory function was very high, with ICC ranging from 0.956 to 1.00. SEM% was between 0% and 3.6% of the relative mean value. Similarly, parameters for HRV evaluation ranged from 0.940 to 0.972, for ICC with SEM% between 3.2% and 7.6%. Lastly, variables for endothelial function assessment were very high for baseline and peak diameter (ICC: 0.998 – 0.999, respectively) and low for SR (ICC: 0.603) with SEM% between 0.4% and 9.2%. The $MDC_{95\%}$ calculated are also presented in Table 2. All variables presented percentage post-RMT variations higher than those required by $MDC_{95\%}$.

Table 2 Reliability and sensitivity of the main measurements.

	Parameter	Trial 1	Trial 2	ICC	SEM%	$MDC_{95\%}$
Pulmonary function	FVC (l)	4.00 ± 0.79	3.98 ± 0.79	0.999	0.6	2
	FEV_1 (l)	3.28 ± 0.62	3.26 ± 0.68	0.999	0.6	2
	MIP (cmH_2O)	49.83 ± 6.71	49.83 ± 6.71	1.00	0.0	0
	MVV ($l \cdot min^{-1}$)	127 ± 22	132± 24	0.956	3.6	10
	RV (l)	0.70 ± 0.12	0.71 ± 0.11	0.978	2.3	6
Heart rate variability	HR rest ($beats \cdot min^{-1}$)	66 ± 8	64 ± 9	0.940	3.2	9
	LF (n.u.)	46.20 ± 20.51	45.98 ± 19.78	0.970	7.6	21
	HF (n.u.)	53.70 ± 21.67	53.92 ± 20.63	0.972	6.6	18
Endothelial function	Baseline diameter (cm)	0.29 ± 0.04	0.29 ± 0.05	0.998	0.7	2
	Peak diameter (cm)	0.32 ± 0.04	0.31 ± 0.04	0.999	0.4	1
	Cumulative SR (s^{-1})	288894 ± 38565	297529 ± 47360	0.603	9.2	26

FVC, forced vital capacity; FEV_1, forced expiratory volume during the first second of the test; MIP, maximal inspiratory pressure; MVV, maximal voluntary ventilation; TLC, total lung capacity; RV, residual volume; LF, low frequency; HF, high frequency; HR, heart rate; n.u., normalized unit. ICC, interclass correlation coefficient; SEM, Standard Error of Measurement; MDC, minimum detectable change. Data are expressed as mean ± SD.

Pulmonary function

As shown in Table 3, MIP and MVV increased significantly only in the RMT (+31% and 16%, respectively; P<0.05).

Table 3 Pulmonary function parameters.

	RMT				SHAM			
	Pre	%Pred	Post	%Pred	Pre	%Pred	Post	%Pred
FVC (l)	4.10 ± 0.14	101 ± 3	4.15 ± 0.16	102 ± 3	3.91± 0.2	103 ± 4	3.88 ± 0.2	104 ± 6
FEV_1 (l)	3.37 ± 0.13	97 ± 2	3.45 ± 0.13	99 ± 2	3.18 ± 0.2	98 ± 3	3.17 ± 0.2	99 ± 3
MIP (cmH_2O)	48 ± 4		63 ± 5*†		52 ± 2		52 ± 2	
MVV ($l \cdot min^{-1}$)	134 ± 4	119 ± 2	155 ± 4*†	131 ± 3	121 ± 6	116 ± 6	125 ± 7	108 ± 4
TLC (l)	4.71 ± 0.14		4.74 ± 0.14		4.70 ± 0.3		4.70 ± 0.3	
RV (l)	0.61 ± 0.07	77 ± 10	0.59 ± 0.05	74 ± 8	0.79 ± 0.05	103 ± 3	0.80 ± 0.04	104 ± 4

%Pred, percent predicted; FVC, forced vital capacity; FEV_1, forced expiratory volume during the first second of the test; MIP, maximal inspiratory pressure; MVV, maximal voluntary ventilation; TLC, total lung capacity; RV, residual volume. Data are expressed as mean ± SE. *P<0.05 *vs* Pre. RMT, respiratory muscle training group; † P<0.05 Post-RMT *vs* Post-SHAM.

Heart rate variability

No differences in resting HR and HRV components (LF and HF) were found, as shown in Table 4. Similarly, the LF/HF ratio did not change after RMT.

Table 4 Heart rate variability data.

	RMT		SHAM	
	Pre	Post	Pre	Post
LF (n.u)	44.66 ± 4.90	40.69 ± 4.95	47.65 ± 6.11	47.43 ± 5.78
HF (n.u)	55.41 ± 4.89	57.64 ± 5.45	52.06 ± 6.06	52.27 ± 5.74
LF/HF	1.00 ± 0.19	0.87 ± 0.22	1.36 ± 0.36	1.28 ± 0.32
Resting HR (beats·min^{-1})	65 ± 4	64± 3	68 ± 2	65 ± 3

HRV, heart rate variability; LF, low frequency; HF, high frequency; HR, heart rate; n.u, normalized unit; RMT, Respiratory muscle training group. Data are expressed as mean ± SE.

FMD and reactive hyperemia assessments

No differences in brachial artery diameter prior to inflation were observed, either pre-or post-training in RMT and SHAM groups (RMT: 0.30 ± 0.01cm *vs* 0.31 ± 0.01 cm, P>0.05; SHAM: 0.28 ± 0.01 cm *vs* 0.28 ± 0.01 cm, P=n.s). Although the percent change in FMD was not found to be significant (Figure 13, panel A), a significant increase in peak brachial artery diameter following cuff release (Figure 13, panel B) was found in the RMT group (P<0.05). There were no differences in the time to peak diameter change between pre-and post-intervention in both groups (RMT: 35 ± 3 s *vs* 28 ± 3 s, P=n.s.; SHAM: 28 ± 2s *vs* 32 ± 3 s,

P=n.s.; pre *vs* post, respectively). We found that SR was lower post-RMT (P<0.05), which was also lower than post-SHAM shear stress response (Figure 13, panel C; P<0.05). SR percentage means difference and CI was: 25% (-10%; 73%) and -3% (-15%; 9%) for pre- vs post- RMT and Sham comparison, whereas 41% (-55%; -3%) between group post data. Consequently, brachial artery FMD was normalized for cumulative SR (%FMD/SR) and post-RMT %FMD/SR increased by 45% (Figure 13, panel D; P<0.05).

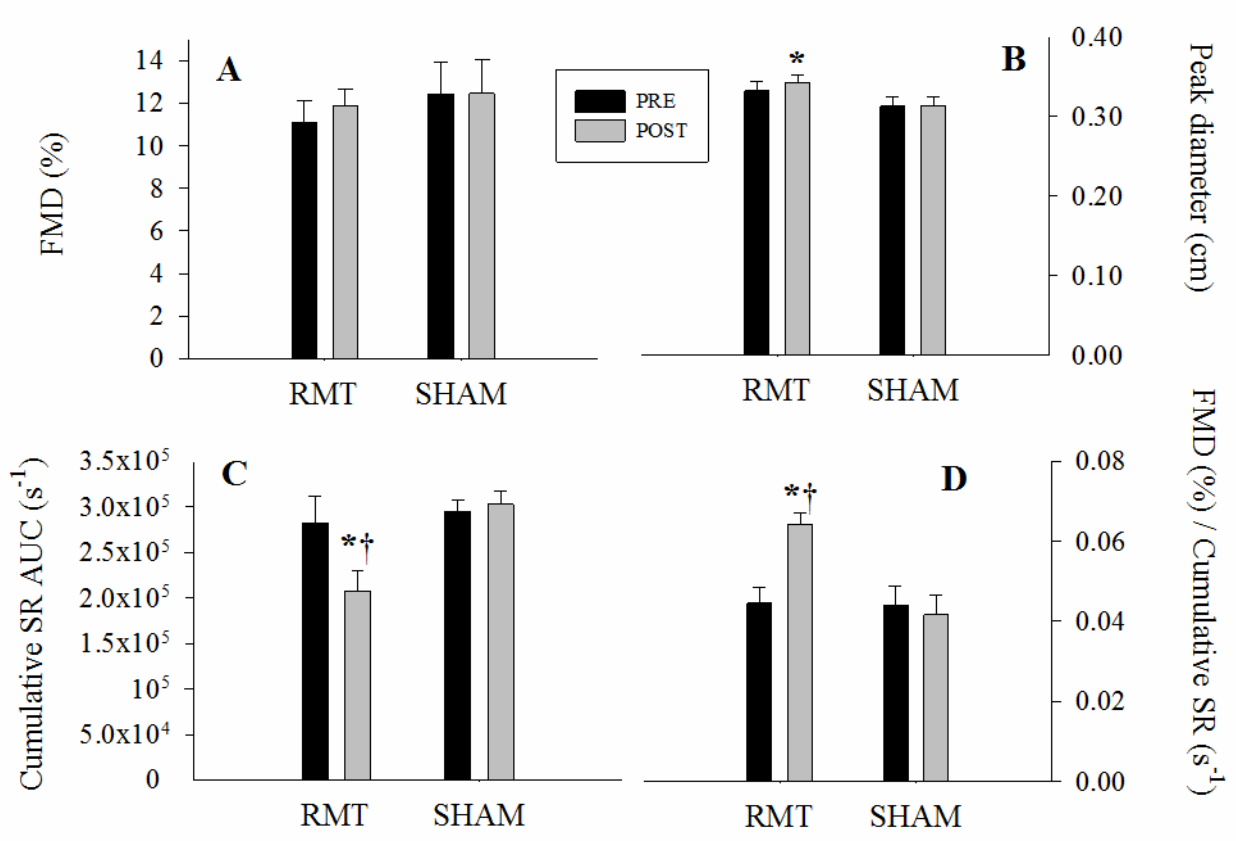

Figure 13. Brachial artery flow-mediated dilation (FMD), expressed as percentage change from baseline (panel A), absolute values at peak diameter (panel B), cumulative SR (panel C), and brachial artery FMD normalized for cumulative SR (panel D) are shown. RMT, respiratory muscle training. Data are expressed as mean ± SE. *P<0.05 *vs* Pre; † P<0.05 *vs* Post SHAM.

Although baseline brachial artery blood flow was not different between RMT and SHAM (Figure 14, panels A and B), the hyperemic blood flow response, after the cuff release, during the FMD maneuver was blunted in post-RMT from second 4 to 80 after cuff release and then returned to baseline levels (Figure14, panel A). This response was not seen in SHAM (Figure 14, panel B). Consequently, the blood flow AUC post-training was 29% ($P<0.05$) lower than pre, only in RMT but not in SHAM (Fig. 3, panel C).

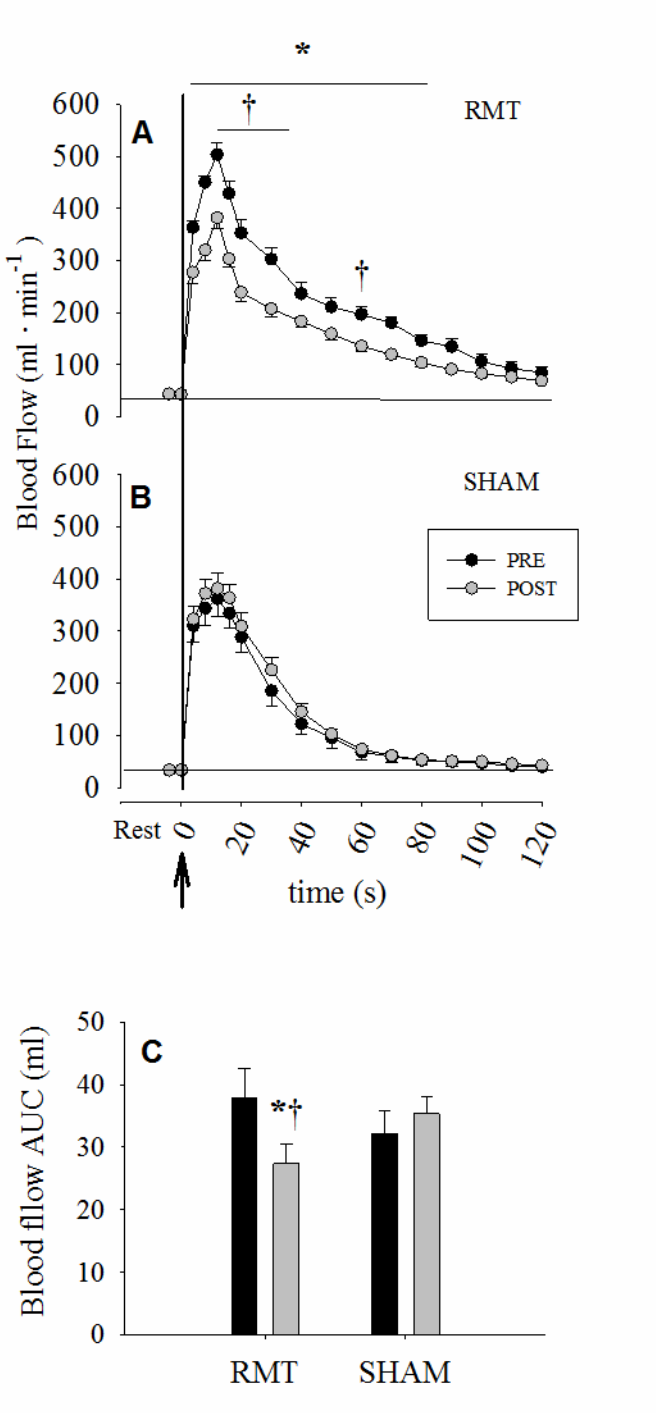

Figure 14. Post occlusion reactive hyperemia, expressed as absolute blood flow (panel A and B) and area under the curve (AUC, panel C) in RMT (respiratory muscle training) and SHAM group. The ↑ in proximity to zero represents the exact time of cuff release. Data are expressed as mean ± SE. *P<0.05 *vs* Pre. †P<0.05 *vs* Post SHAM.

Chapter 5 – Study 2

Effects of Isolated Muscle Training on Vasomotor Response and Peripheral Blood Flow

5.1 Materials and methods

5.1.1 Participants

Ten young participants (Table 5) volunteered for study. Exclusion criteria were: (i) presence of neurological, vascular and musculoskeletal impairment at the lower and upper limbs level; (ii) being on medication therapy that could alter or change the neural and/or vascular response including hormonal contraceptives and oral supplements; (iii) smokers; (iv) systolic blood pressure higher than 140 mmHg; and (vi) irregular menstrual cycles (26 to 35 days). The Institutional Review Board approved the study, which was performed in accordance with the principles of the latest Declaration of Helsinki.

Table 5 Participants' characteristics.

Participants (n. M/F)	10 (4/6)
Age (years)	23 ± 3
Stature (cm)	170 ± 11
Body mass (kg)	66 ± 11
Body mass index (kg/m^2)	23 ± 1

Data are expressed as mean ± SD.

5.1.2 Study design

Before and after 8 weeks of isolated quadriceps muscle training (IQT), participants were tested at the same time of the day in a climate-controlled laboratory (constant temperature of 20 ± 1 °C and relative humidity of 50 ± 5 %). For the female included in the study, the tests were assessed in the same day of the menstrual cycle (early luteal phase). Female participants recorded their menstrual cycle in a personal diary throughout the eight weeks of the study. Then, it was used to assess the early luteal period. On the test~~s~~ day, subjects came to the laboratory after fasting overnight, abstaining from caffeine and other similar substances for at least 12 hours, and not involved in heavy exercise for 48 hours. Post interventional experiments were performed 24 to 48 hours after the last IQT session to prevent examination of the acute effects of training. During IQT first session, the possible increase in brachial artery blood flow was also assessed.

5.1.3 Measurements

Maximum work rate (MWR)

MWR was determined on a dynamic knee extension (KE) ergometer model (Anderson&Saltin, 1985) (Andersen et al. 1985) by an incremental square wave test. This test was performed sitting on chair, in which the back support was mobile in order to fit legs of different lengths. Both knees were flexed at 90° and one ankle was attached by a rigid bar to a pedal arm of a bicycle ergometer, which could be connected to the chair for a major stability. A complete active knee extension movement (quadriceps muscle work), from 90° to 170°, and a passive flexion using a flywheel momentum, corresponding to a complete turn of the pedal arm. The mechanical brake applied to the ergometer and the revolutions per minute (rpm) determined the work

performed. Square wave test started with a load of 20 W for males and 10 W for females. Loads increased by steps of similar amplitude (+20W for male and +10W for female) until exhaustion. Each load was maintained for 3 minutes with 5 minutes of recovery in between. The load of last completed step was considered as MWR. A non-invasive impedance cardiograph device, Physio Flow® (Manatec Biomedical, Paris, France) was used to assess central hemodynamics parameters as cardiac output (CO), stroke volume (SV) and heart rate (HR). Data were measured second-by-second and presented as average of the last 60 s of baseline and last 30 s of the maximum load reached

Single Passive limb movement (PLM)

PLM was performed in accordance to the recommended procedures (Trinity et al. 2012; Venturelli et al. 2017). Sitting on KE ergometer, subjects rested in the upright-seated position for 10 min before starting data collection and remained in this position throughout the end of the test. The PLM protocol consisted of 60 s of resting baseline peripheral hemodynamic data collection, followed by a single passive knee flexion and extension, which took 1 s, after which the leg was maintained fully extended for the remaining 59 s of post-movement data collection. PLM was performed by a member of the research team, who moved the subject's lower leg through a 90° range of motion (180 –90° knee joint angle) at 1 Hz. Throughout all the PLM test, measurements of arterial blood velocity and vessel diameter were performed in the common femoral artery of the passively moved leg, distal to the inguinal ligament and proximal to the deep and superficial femoral bifurcation with a Logiq-7 ultrasound Doppler system (General Electric Medical Systems, Milwaukee, WI). A 9 -MHz linear array transducer was used. Blood velocity (V_{mean}) was measured using the same probe utilizing a frequency of 5

MHz. Measurements of V_{mean} were obtained with the probe positioned to maintain an insonation angle of 60° or less, and the sample volume was centered and maximized according to vessel size (Trinity et al. 2012). Femoral artery blood flow was calculated by using data of arterial diameter and mean blood velocity (v_{mean}) and as:

$$\text{Blood flow (BF, ml} \cdot \text{min}^{-1}) = v_{mean} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60$$

Where BF was in milliliters per minute.

Flow mediated dilation (FMD)

Brachial artery FMD measurements were performed according to recommended procedures (Harris et al. 2010). Before FMD, the subjects laid supine for approximately 20 minutes to restore baseline values of cardiovascular parameters. A blood pressure cuff was placed on the forearm immediately distal to the olecranon process in order to provide a stimulus for forearm ischemia when inflated. Following baseline assessments, the forearm blood pressure cuff was inflated to 250 mmHg for 5 min. Diameter and flow velocity recordings resumed at baseline, 30 s prior to cuff deflation and continued for 2 min post-deflation in accordance with recent technical specification (Corretti et al. 2002; Harris et al. 2010; Wray et al. 2013). A 12- to 14-MHz linear array transducer, attached to a high-resolution ultrasound machine (Vivid I, GE Medical Systems, Milwaukee, WI, USA) was used to image the brachial artery in the distal third of the upper arm. When an optimal image was obtained, the probe was held stable and longitudinal B-mode images of the lumen-arterial wall interface were acquired. Continuous Doppler velocity assessments were also obtained using the ultrasound and were collected using the lowest possible insonation angle (always <60°). The FMD data were exported in AVI format and analyzed using commercially available software (Brachial Artery Analyzer for Research,

Medical Imaging Applications, LLC, Coralville, IA), which is largely independent of investigator bias. FMD was quantified as the maximal change in brachial artery diameter after cuff release, expressed as a percentage increase (%Δ) above baseline:

(Peak – baseline diameter) / baseline diameter · 100.

Brachial artery BF was calculated as previously described for PLM assessments.

The shear rate (SR) was calculated post cuff release using the following equation:

$$SR\ (s^{-1}) = 8\ v_{mean} / \text{vessel diameter}$$

The cumulative SR, corresponding to the reactive hyperemia post cuff release (total SR from cuff release to time to peak), was integrated (area under the curve, AUC) by using the trapezoidal rule, and calculated as:

$$\Sigma[yi \cdot (x \cdot (i\text{-}i) - xi) + (1/2)\ (y\ (i\text{-}i) - yi)\ (x\ (i\text{-}i) - xi)]$$

The cumulative/integrated SR (AUC) reflects the amount of mechanical stimulus applied on the endothelium during the cuff release hyperemic response at time to peak. Considering that FMD is primarily dependent on the endothelium response to mechanical stimuli, the %FMD was therefore divided by cumulative SR (%FMD/SR) (Pyke and Tschakovsky 2005).

Isolated quadriceps muscle training (IQT)

A schematic drawing of the experimental design is given in Figure 15.

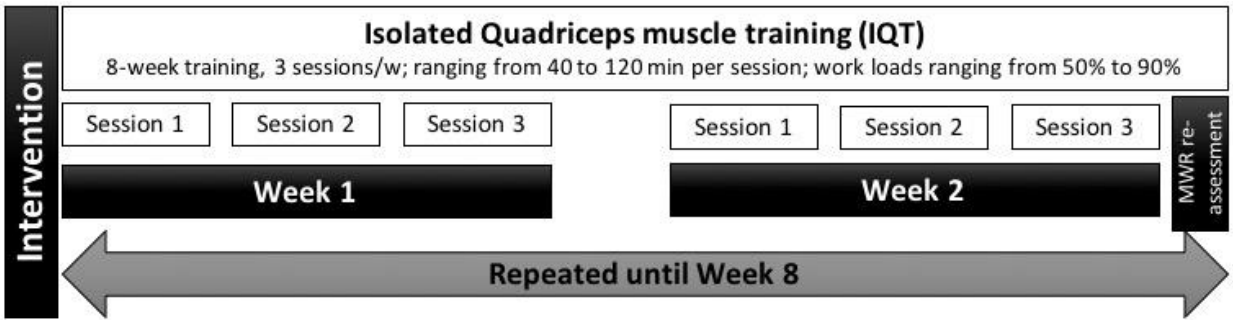

Figure 15. A schematic drawing resuming the study design.

Participants underwent an 8-weeks IQT (3 sessions/w) involving both legs. Exercise training was performed on an Anderson&Saltin ergometer. This ergometer allows training only the leg extensor muscles of a single limb under an aerobic regime. The IQT will be similar to a previous study (Esposito et al. 2010c). Briefly, workloads ranged from 50% to 90% MWR with averaged session duration of 40 mins for each leg. Each training sessions were supervised by an expert operator, who monitored both the attendance and correct exercise execution. The sessions workloads were readjusted every two weeks performed a new MWR. The participants not attending at least the 80% of training were excluded from the study. In this case, a new participant was recruited to substitute the drop out.

During the first session of IQT (performed at 50% of MWR), to assess the acute effects of exercise on brachial artery BF and SR and central parameters such as heart rate, stroke volume and mean arterial pressure. During this session, continuous Doppler brachial blood

velocity and vessel diameter were assessed by ultrasound. HR (bpm) and mean arterial pressure (MAP, mmHg) were determined by electrocardiography and by finger photoplethysmography (Finometer PRO, Finapres Medical System, Amsterdam, The Netherlands) positioned at the heart level, respectively. SV (ml) was automatically calculated using the Modelflow method (Finapres Medical System), with CO ($l \cdot min^{-1}$) calculated as the product of SV and HR. Before starting the bout of exercise training, a baseline measurement of 30 seconds was recorded. The brachial blood velocity and brachial artery diameter assessments were then used to calculated the brachial BF (BA BF) ($ml \cdot min^{-1}$).

Statistical analysis

Statistical analysis was performed using a statistical software package (IBM SPSS Statistics v. 22, Armonk, NY, USA). Shapiro-Wilk test was used to check the normal distribution of the sampling. A sample size of 10 participants was selected to ensure a statistical power higher than 0.80 based on a Post-Hoc: compute achieved power given the effect size and the simple size of maximum BF during PLM using G*Power software (Faul et al. 2007). To assess significant effects of IQT on MWR, PLM and FMD parameters, a paired Student's t-test was applied pre and post data. A two-way ANOVAs for repeated measures (training x time) were applied to disclose possible differences in the BF kinetics during PLM hyperemic response. Statistical significance was accepted at $P<0.05$. Values are expressed as mean ± standard deviation (SD). Additionally, the same results were interpreted using also meaningful differences in a magnitude-based approach for analysis and reporting (Hopkins 2000). Pre-post change scores with confidence intervals for each parameter of each tests are reported. Effect sizes measure expressed as Cohen's *d* was calculated for each parameter to quantify within-group magnitude

changes (Cohen 1992). Values of 0.2, 0.6 and 1.2 were used to determine if the effect sizes (magnitude of change) were small, medium and large respectively (Page 2014).The percent likelihood that the observed effect size was larger than the smallest worthwhile change was calculated based on previous methods (Hopkins 2000; Page 2014). Chances of a meaningful difference was classified as follows: <1%, almost certainly not; <5%, very unlikely; <25%, unlikely; 25–75%, possible; >75%, likely; >95%, very likely; >99% almost certain. The ≥75%, likely, classification was used as the threshold for a meaningful difference (Page 2014).

5.2 Results

Central hemodynamic parameters and peripheral blood circulation assessed acutely during the first session of IQT, performed at 50% of MWR, are presented in Figure 16.

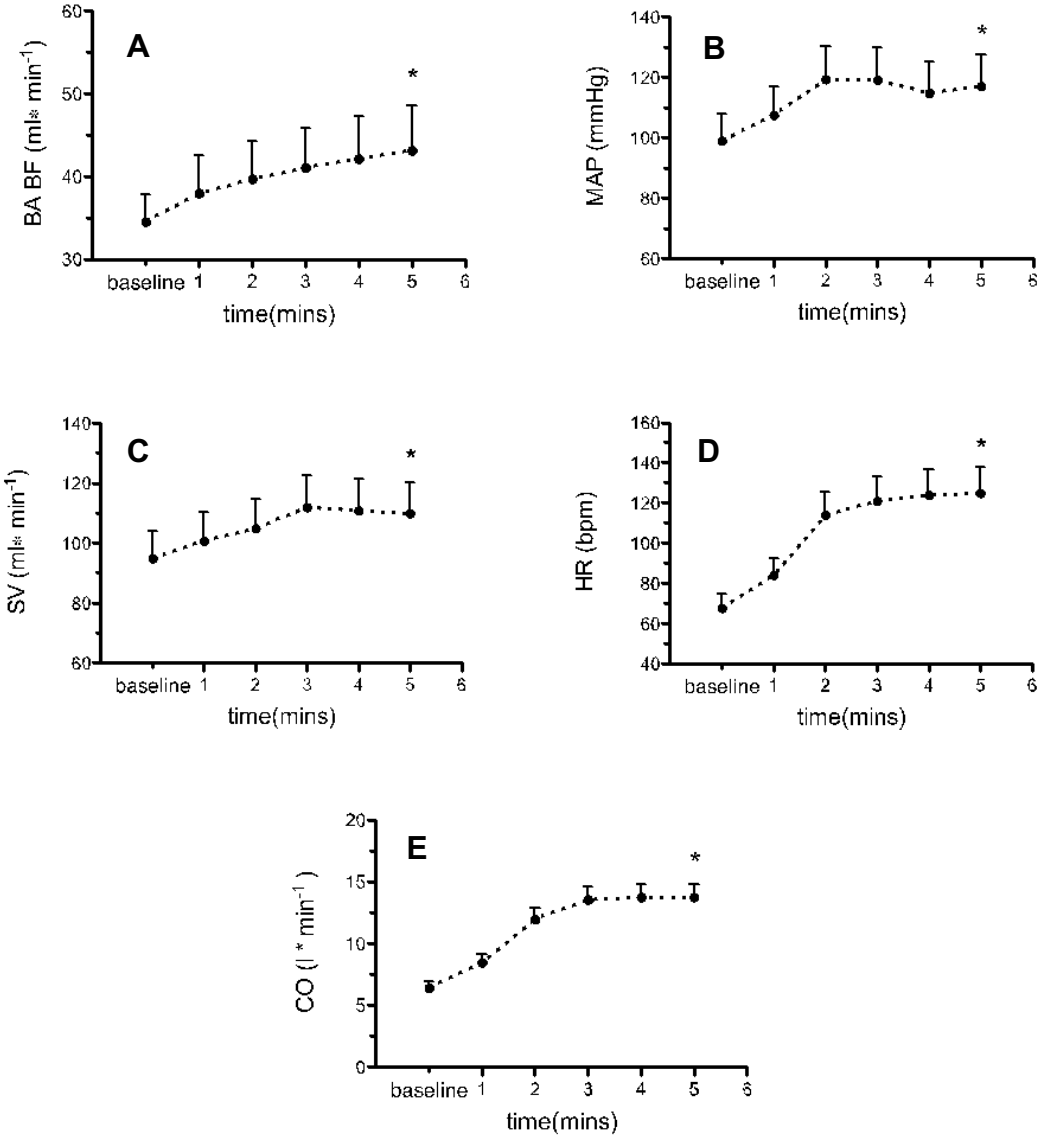

Figure 16. Brachial artery BF during IQT (BA BF, panel A), mean arterial pressure (MAP, panel B), stroke volume (SV, panel C), heart rate (HR, panel D) and cardiac output (CO, panel E) are shown. Data are expressed as mean ± SD. *P<0.05 *vs* baseline.

All the parameters result to be significantly higher at the end of the 5th minute of exercise compared to baseline. Specifically, BA BF and MAP increased from 35 ± 3 to 43 ± 5 ml · min^{-1} and 99 ± 9 to 117 ± 11 mmHg, + 25% and +18% respectively. SV and HR increased from 95 ± 5 to 110 ± 10 ml · min^{-1} and 68 ± 7 to 125 ± 13 bpm, + 16% and + 114% respectively, leading to CO increment from 6 ± 0.5 to 14 ± 1.0 l · min^{-1}, +85%.

Maximum work rate (MWR)

As shown in Table 6, MWR significantly increased after 8 weeks of IQT (+ 44 %, P < 0.001). Differently, no differences were found in central hemodynamics parameters, such as SV, HR and CO after IQT.

Table 6. Maximum work load and relative central hemodynamics parameters

	PRE	POST	*P* value
MWR (w)	48 ± 13	69 ± 21	<0.001
SV_{rest} (ml · min^{-1})	77 ± 37	84 ± 22	0.66
SV_{max} (ml · min^{-1})	100 ± 43	106 ± 28	0.74
HR_{rest} (bpm)	72 ± 8	72 ± 8	0.99
HR_{max} (bpm)	148 ± 8	147 ± 19	0.91
CO_{rest} (l · min^{-1})	5 ± 2	6 ± 1	0.42
CO_{max} (l · min^{-1})	15 ± 7	16 ± 4	0.84

MWR, Maximum work rate; SV, stroke volume; HR, heart rate; CO, cardiac output. Data are expressed as mean ± SD.

Single Passive limb movement (PLM)

BF response to PLM is illustrated in Figure 17 and Figure 18.

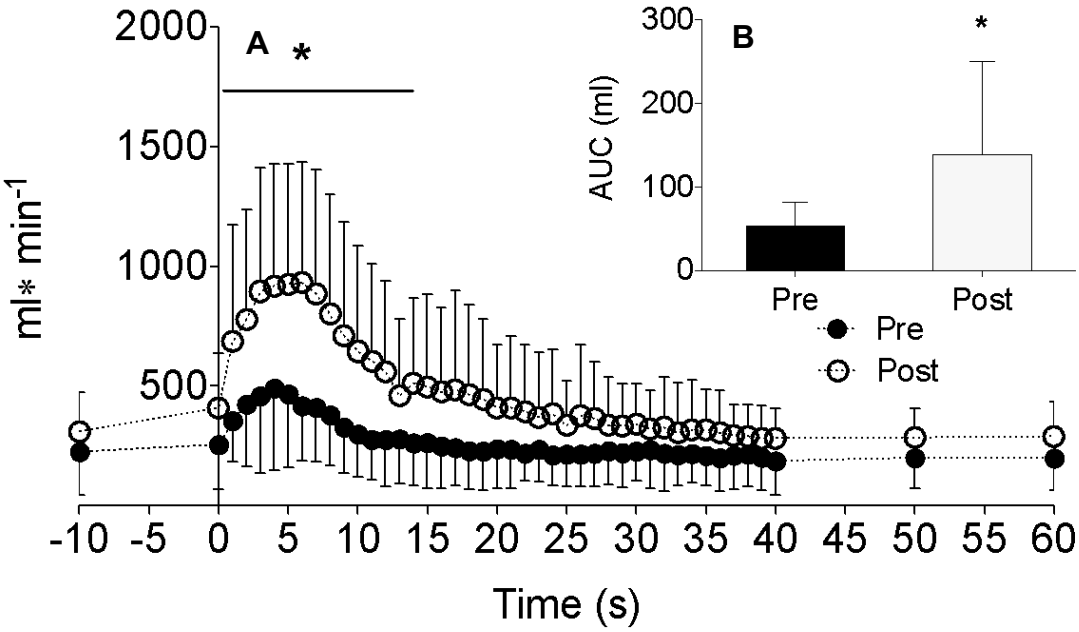

Figure.17. The hyperemic leg blood flow kinetics (panel A) and its relative blood flow AUC (panel B) response to PLM. Values are means ± SD. *Significantly different Pre vs Post $P<0.05$.

PLM baseline BF was similar, 225 ± 180 and 309 ± 163 ml · min^{-1} pre vs post respectively ($P=0.30$ Figure 17, panel A). Conversely, a significantly increase in BF was found from the 2nd to 14th second of the PLM BF kinetic post IQT ($P<0.05$, Figure 17, panel A). PLM AUC was higher after IQT, from 53 ± 28 to 138 ± 112 ml (+160% post vs pre, $P<0.05$; Figure 17, panel B). Similarly, BF_{max} and ΔBF were greater after IQT, from 528 ± 318 to 1078 ± 505 ml · min^{-1} (+ 104% post vs pre $P<0.05$; Figure 18, panel A) and 304 ± 158 to 769 ± 399 ml (+ 152 % post vs pre $P<0.05$; Figure 18, panel B) respectively.

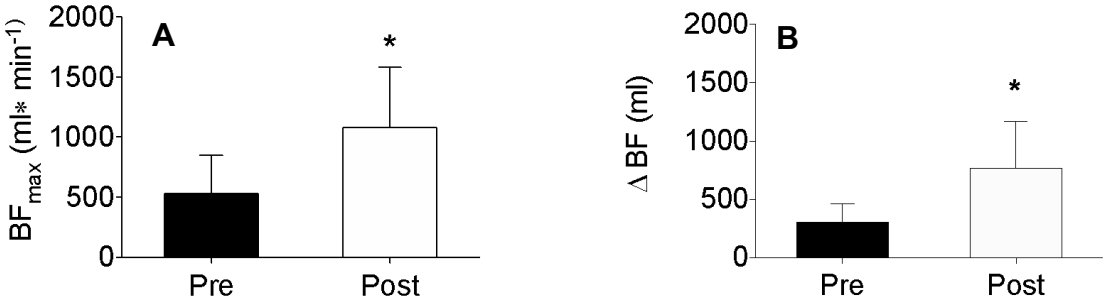

Figure 18. Maximum BF (panel A) and Δ BF (BF_{max} - $BF_{baseline}$; panel B) in the PLM response pre vs post IQT. Values are means ± SD. *Significantly different Pre vs Post $P<0.05$.

Data were reanalyzed using magnitude-based approach confirming the results previously presented for the same PLM's parameters (Figure 19). Indeed, PLM AUC, BF_{max} and Δ BF, significantly higher after IQT with the t-test approach, were also found to have a very like probability that the magnitude of the standardized difference between pre and post IQT was higher than the minimal detectable change (99%, 99% and 98% likelihood respectively).

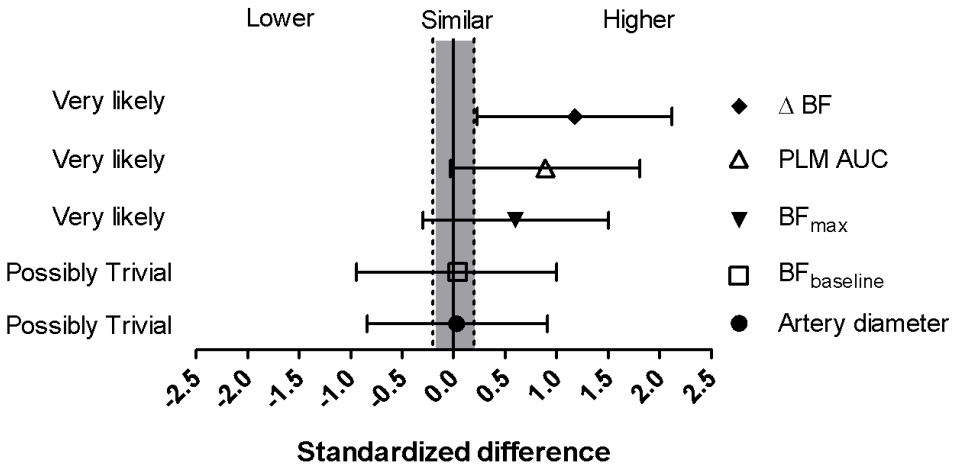

Figure 19. Magnitude based-inferences model applied to PLM's parameter (femoral artery). Data are presented as standard exercise effect size with IC95%. In the left side column, the likelihood that the difference between pre- and post- training measurement is higher than the minimum detectable change is provided.

Flow mediated dilation (FMD)

FMD data were summarized in table 7. No significant differences were detected after 8 weeks of IQT in any parameter.

Table 7. Flow Mediated Dilatation (FMD) data pre vs post IQT.

	PRE	POST	*P* value
FMD (%)	19 ± 9	18 ± 9	0.93
Peak Diameter (mm)	3.5 ± 0.8	3.7 ± 0.8	0.25
Time to Peak (s)	27 ± 11	26 ± 8	0.83
SR at Peak (s^{-1})	2879 ± 1085	3997 ± 1923	0.20
FMD/SR (%/ s^{-1})	0.06 ± 0.03	0.05 ±0.03	0.86
FMD BF AUC (ml)	31 ± 15	41 ± 26	0.14

FMD, flow mediated dilatation; SR, shear rate; BF AUC, blood flow area under the curve. Data are expressed as mean ± SD.

FMD magnitude based-inferences analysis revealed similar results for all the parameter except for FMD BF AUC (Figure 20). On the contrary to the standard statistical approach, FMD BF AUC resulted to have a very likely probability that the effect size calculated is higher than the minimal detectable change.

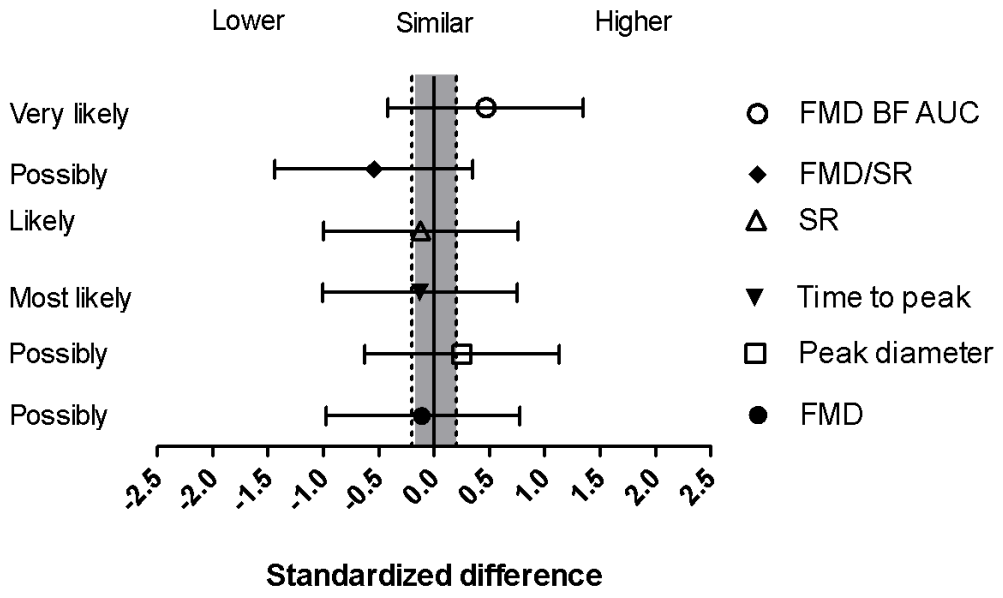

Figure 20. Magnitude based-inferences model applied to FMD's parameter (brachial artery). Data are presented as standard exercise effect size with IC95%. In the left side column is the likelihood that the difference between pre- and post- training measurement is higher than the minimum detectable change is provided.

Chapter 6- Discussion

Aim of this dissertation was to investigate the vascular plasticity-effects, in terms of functional and structural modifications, induced by small muscle exercise training. On this purpose two different studies were achieved using two different small muscle exercise training models: RMT and IQT. In the first, RMT was used in order to affect both components of the vasomotor response: global control, driven by the sympathetic nervous activation (measured by HRV), and peripheral control, related to the endothelial cells ability to respond to stimuli and measured by FMD. In the second study, IQT was used to shed a light in how wide could be the beneficial effect on the vasculature related to the peripheral component. Specifically, the IQT vasculature effects were assessed at two different levels, in the vasculature of muscles directly involved during exercise, by PLM technic (femoral artery), and distantly from the exercising point by FMD technic (brachial artery).

The results showed that: (i) RMT had a positive effect on the FMD without affecting HRV, and (ii) IQT had a positive effect on the PLM without any changes in the FMD. This suggests that both small muscle training models, RMT and IQT, positively influenced the vasomotor response in its peripheral component, and these beneficial effects are exercise intensity dependent.

The effectiveness of both RMT and IQT were confirmed by the improvements in the pulmonary function, as indicated by MVV, MIP, and the MWR respectively. Such results were qualitatively expected and well in agreement with previous findings (Hepburn et al. 2005; Verges et al. 2009; Esposito et al. 2010b, a, 2011; Wolff et al. 2018).

6.1 Effects of small muscle training on the global control of the vasomotor response

The exercise training concept can be applied to every single muscle belonging to the locomotor system, including the respiratory muscles. RMT is a small-muscle exercise model generally employed to improve pulmonary function (Esposito et al. 2010b, a) as well as respiratory muscle endurance and strength (Faghy and Brown 2016). In presence of some pathologies, such as chronic heart failure, RMT could be beneficial for many physiological aspects, such as changing the respiratory patterns at rest (Faghy and Brown 2016). It is important to underline that chronic heart failure is characterized by dyspnea, exercise intolerance (leading to physical deconditioning), abnormalities in the myocardial pump function and autonomic nervous system impairment (i.e. a higher sympathetic activation) (Faghy and Brown 2016). In this population, a reduction in the sensitivity of the chemo-, baro- and ergo-reflex, which are cause of hyperventilation and vasoconstriction, is the result of changes in the respiratory patterns due to a direct effect in rebalancing the neural autonomic activation (Faghy and Brown 2016). Moreover, in heart failure and hypertensive patients this autonomic rebalancing (i.e. decrease in the sympathetic activation), results, after RMT, in an increase in the peripheral blood flow via a reduction in peripheral resistances (Chiappa et al. 2008; Ferreira et al. 2013). Interestingly, changes in the neural autonomic activity, assessed by HRV, where an increase HF and a decrease LF power is recorded, were also described after RMT in a healthy population (Hepburn et al. 2005). This led to the idea that blood flow adaptations may occur similarly to what happens in diseases population.

Whole body endurance training, such as cycling or running, have many positive effects on the cardiovascular system such as an increase in the left ventricular internal dimension, wall thickness, and in the end-diastolic volume, leading to an increase in stroke volume that allows

for a lower HR at rest (Bellenger et al. 2016). Specifically, HR modification at rest seems to be linked to an increase in HF peak power of HRV and potentially reflecting a higher parasympathetic activation (Bellenger et al. 2016). Conversely, in the present study no significant changes were detected in resting HR and in LF or HF (see Table 4), even though a significant difference were found in MVV (see Table 3), an index of respiratory muscle endurance performance, raising the question if RMT offered a stimulus strong enough to achieve the abovementioned central adaptations.

Nevertheless, the literature provides some evidences suggesting that RMT produces similar benefits, in terms of vagal activation, without affecting HR at rest (Hepburn et al. 2005). Indeed, Hepburn et al., studying the HRV after RMT, discovered that an increase in HF component, an index of vagal tone activity, was found, together with a significant decrement in the LF, index of sympathetic activation (Hepburn et al. 2005). Hepburn et al. data dare not in line with the findings obtained in the present investigation (see Table 4). Differences in the methodological approach used and in the population characteristics could likely explain the dissimilarities between the two studies. Indeed, Hepburn and co-workers investigated the RMT-effects in an older population compare to the study presented in this dissertation (52 ± 4 *vs* 25 ± 9 years, respectively), and with baseline values of LF and HF different (LF 49.9 ± 6.2 and HF 41.7 ± 5.7 *vs* LF 44.66 ± 4.90 and HF 55.41 ± 4.89, respectively). Interestingly, disparities in HRV frequencies at baseline, as a consequence of aging, lead RMT in Hepburn's study to be more effective (i.e. on the LF of the HRV components) and consequently positively affect sympathetic and parasympathetic activity.

Indeed, other prior studies demonstrated that RMT not only rebalance the autonomic response in ageing people (Hepburn et al. 2005), but also in patients with hypertension and heart failure

(Porta and Bernardi 2001; Chiappa et al. 2008; Ferreira et al. 2013; Faghy and Brown 2016). Ageing, hypertension and heart failure are all characterized by non-uniform impairment in sympathetic and parasympathetic tone, where an over expression of the sympathetic drive is recognized (Umetani et al. 1998; Porta and Bernardi 2001; Chiappa et al. 2008; Ferreira et al. 2013). Therefore, it is tempting to speculate that the positive effects of RMT on HRV may be only observable in a population already characterized by alterations of the sympatho-vagal balance.

6.2 Effects of small muscle training on the local control of the vasomotor response

Either basal vascular and vasomotor tone refer to the amount of smooth cells contraction necessary to maintain the vessels partially constricted (Rhodin 1980), because of the low oxygen consumption in skeletal muscle under resting conditions (Korthuis 2011a; Joyner and Casey 2015). However, during physical activity, an enormous increase in skeletal muscle blood is necessary to meet the 20- to 50-fold increase in the demand for oxygen and substrates that are required to support the markedly enhanced metabolic activity of the active muscles (Korthuis 2011a, b). At the peripheral level, vascular resistance is determined by blood viscosity, tissue vascularization (i.e., the size and number of vessels), and the caliber of the resistance vessels (Korthuis 2011a, b). Considering blood as "viscosity constant", at least over the short term, it could be stated that alterations in skeletal muscle blood flow are regulated by modulating the diameter of resistance arteries and arterioles by smooth muscle cells contraction (Korthuis 2011a, b). More specifically, vasomotor tone is the change in the caliber of the resistance arteries elicited by alterations in the contractile activity of smooth muscle cells from the basal level (Korthuis 2011a, b). Moreover, following the Poiseuille's law, flow (Q) is related to the perfusion pressure (P), and the radius (r), length (l), and viscosity (η) of the vessel:

$$\dot{Q} = \frac{\Delta P \pi r^4}{8 \eta l}$$

Vascular resistance varies inversely with fourth power of the radius meaning that small changes in vessel caliber produce very large variation in blood flow. Therefore, it seems clear how important is to have an efficient vasomotor response throughout the arterial tree.

As already mentioned, the vasomotor response is primarily controlled by two different control mechanisms: central (or remote) control mechanisms, mediating vasoconstriction by

sympathetic neural activation; and local influences, intrinsic to the tissues and related to endothelial cells activity mediating the relaxation of smooth muscle cells (Korthuis 2011b).

The present dissertation aimed to investigate the effect of small muscle exercise training on the local component of the vasomotor response. Considering that the exercise training is used as a tool to increase the peripheral circulation, it was hypothesized that the main mechanism acting on this local component, i.e. the endothelial cell reactivity, could be ascribed to the shear stress stimulus.

As previously described, the shear rate is the drag force acting on inner vessel that mediated the release of vasoactive factors by the endothelial cells (Niebauer and Cooke 1996). Indeed, blood flow oscillation, such as changes in blood flow pattern during every single bout of exercise, may be an important stimulus to endothelial cell membrane deformation and the consequent signaling-events favoring NO production (Green et al. 2005). It was also previously demonstrated by Thoma that a repetitive bouts of higher blood velocity in a tissue leads to angiogenesis (Thoma R 1893). Additionally, Rodbard proposed the model of prediction flow-dependent, endothelium-mediated dilation and remodeling (Rodbard 1975). According to this model, changes in the blood flow, occurring in the vessels, induce not only an acute vasodilation (i.e improvement in vascular functionality), but eventually, the chronic repetition of this stimulus, can also promote a vascular remodeling (i.e. change in the vasculature structure) (Rodbard 1975).

Lastly, it seems that the positive effects of the shear rate on the vascular functionality are strictly dependent on the magnitude with which the shear rate stimulus acts on the vessel (Green et al. 2011).

The effect of small muscle training in vasculature directly involved during exercise, and thus directly exposes to a greater magnitude of shear stress stimulus, was assessed with PLM after 8 weeks of IQT. IQT involved just the quadriceps muscles exercising, and the PLM-test assessed the vascular functionality in the common femoral artery. As results of 8 weeks of IQT, all the PLM-related parameters, such as BF AUC, BF max and Δ BF, together with the MWR were significantly improved. This result highlighted the positive effect of this type of training not only on the performance (i.e. MWR) but also on the vasculature directly expose to the exercise. Considering the nature of this hyperemic response, we can ascribe such increment to an enhanced NO-bioavailability (Trinity et al. 2015; Venturelli et al. 2017) To our knowledge this is the first study that investigated the effects of exercise on vascular functionality with PLM. Many other studies reported the positive effect of a small muscle training (i.e. handgrip) on the FMD (Tinken et al. 2010; Credeur et al. 2012; Badrov et al. 2016), which is the most common tools utilized to evaluated non-invasively and in vivo the vascular functionality (Corretti et al. 2002; Harris et al. 2010; Thijssen et al. 2011a). Also in these studies the positive effects of training on the vasculature are mainly imputable to the shear stress stimulus acting on the inner vessel lay and leading to an increase vasodilation capacity (Tinken et al. 2010; Credeur et al. 2012; Badrov et al. 2016), likely due to an increase in the NO-bioavailability (Corretti et al. 2002; Tinken et al. 2010; Harris et al. 2010; Thijssen et al. 2011b, a; Green et al. 2017b). It is therefore necessary underline that the increase in the PLM response, due to a shear stress repeated stimuli, does not lead to an enhancement in the common femoral vasodilatory capacity (Venturelli et al. 2017). The common femoral artery is the largest artery in the thigh, which has the main role to delivery blood flow instead of regulates it. PLM produces a brief and transient movement-induced hyperemia (without the increase in metabolism associated with active

exercise) (Trinity et al. 2012; Venturelli et al. 2017), as a results of a drop in the peripheral resistances and largely NO-mediated (Trinity et al. 2012), that gives an indication about the vascular and microvascular functionality (Rossman et al. 2016). Most important, it was previously demonstrated that the PLM hyperemic response provides an index of vascular function analogous to the traditional FMD test (Rossman et al. 2016).

Interestingly, in the present dissertation the effect of small muscle training (RMT and IQT) was also studied in muscle vasculature not directly involved during exercise. Specifically, the vascular functionality was assessed in the brachial artery after 8 weeks of RMT or IQT by FMD. It was previously shown by Birk et al. that the chronic shear stress stimulus on brachial artery FMD before and after eight weeks of cycle exercise training lead to an enhancement only where the shear rate was unrestrained (Birk et al. 2012). Specifically, in this experimental design it was planned that during each training session, one arm was always partially blood flow restrain by a cuff to mitigate distal shear stress in the non-exercising district, while the other non-exercising arm was freely perfused. FMD results revealed an improved peripheral vascular response only in the free (unrestrained) arm (Birk et al. 2012), confirming the key role of shear stress in this phenomenon.

Considering that the shear rate beneficial effects of the vasculature are magnitude-dependent (Green et al. 2011), assessments of central parameter such as CO, SV, HR, MAP and brachial artery BF, were acutely taken during a single bouts of exercise, to make sure that a real stimulus was provided.

Acute data referring to brachial BF and cardiovascular central parameters during the first session of IQT shown that after 5 minutes all of them are significantly higher than baseline (see Figure 16). More specifically, the brachial artery BF was 20% higher than baseline BF (see

Figure 16A). As far as we know, this is the first time in which the brachial artery BF was measured during IQT. However, it was previously demonstrated, that IQT is a powerful approach to decrease exercise intolerance in patients with central hemodynamic limitation, such as heart failure or chronic obstructive pulmonary disease, because of the less cardiovascular system involvement compare to a whole body exercise (i.e. cycling) (Esposito et al. 2010c, 2011). Interestingly, brachial BF measured during a cycling exercise recorded around 30% increment respect to baseline values (Green et al. 2005), and besides, as above mentioned, a long term cycling exercise training could lead to an FMD enhancement (Birk et al. 2012). In the present study, after IQT no changes were detected in the % FMD. IQT increased peripheral blood flow in districts not directly involved during exercise training to a lower extent (+20 %) compared to large muscle training (+30%), providing a stimulus not sufficient to improve vascular function in the non-trained limb.

Following the same trend, during a single bout of RMT, performed at two different intensities, brachial artery BF was also significantly higher compare to baseline values in both trials (+145% and 241% respectively). However, only the second intensity was strong enough to rise significantly also all the others cardiovascular parameters (i.e. CO, SV, HR and MAP). In detail, the effort in the first acute trial was similar to the RMT intensity kept during the first week of the training protocol. Contrarily, the second intensity represented the minimum intensity increment achieved by everyone throughout RMT. It could be possible that this BF stimulus in area not directly involved during RMT may become even bigger throughout the training. Indeed, also RMT is able to decrease exercise intolerance (Scano et al. 2006; HajGhanbari et al. 2013; Salvadego et al. 2017). Ameliorations in the pulmonary and physical performance could be determined, at least in part, by improvements in respiratory muscles metabolic

efficiency, and by a reduced O_2 cost of respiratory muscles work (Scano et al. 2006; HajGhanbari et al. 2013; Salvadego et al. 2017). Therefore, it seems that RMT could prevent the development of fatigue within the respiratory muscles delaying the effects of some reflex related to the sympathetic vasoconstriction within the other locomotor muscles limiting the oxygen delivery at the peripheral level (Witt et al. 2007). The same mechanism could have an important and positive influence on the central and peripheral hemodynamics leading to a better blood flow redistribution during a single bouts of exercise from respiratory to locomotor muscles (Witt et al. 2007; Salvadego et al. 2017). However, in the present study the significant increments in BF detected in acute during the preliminary screening, can be primarily explained by the increase in MAP, provoking the increase in brachial vascular conductance and therefore in shear stress.

Results after 8 weeks of RMT shown that despite no change in the %FMD between pre- and post- RMT, a significant drop in the cumulative SR mean difference was found only in the RMT group. Cumulative SR is the stimulus triggering the FMD response (see 2.2.4 Flow mediated dilatation) and the latter is proportional to the level of reactive hyperemia. Therefore, we can conclude that this result clearly suggests the efficacy of RMT treatment in readjusting the amount of mechanical stress necessary to exacerbate FMD response. Moreover, this result becomes stronger when the FMD value has been normalized for the cumulative SR and compared to the shame group (see Figure 13D). A positive effect of RMT on the peripheral vasomotor response was unveiled only in the RMT-group.

Together with a consistent drop in the %FMD/cumulative SR, a significant increase in the peak diameter was detected. This observation suggests that after RMT the same percentage change in diameter could be achieved with a lower mechanical stimulus allowing the speculation that

RMT likely induced structure remodeling of the vessels leading to a more compliant system. Indeed, exercise training may affect vascular tissue not only by modifying the function (e.g., %FMD), but also by inducing structural arterial remodeling, such as baseline and peak diameter (Green et al. 2017a). Based on an animal model, it would appear that structural remodeling of the vessels may counteract the endothelial cells response to reactive hyperemia (i.e., %FMD) (Green et al. 2017a). Moreover, studies in humans reported that during a training protocol changes in FMD occurred during the first few weeks of training before returning to pre-training values, often without any change in baseline diameter (Tinken et al. 2008, 2010; Weber et al. 2013), suggesting that structural remodeling of the vessels may have likely occurred. Despite the model utilized (animal *vs* human model), adaptations in terms of vascular remodelling after a training protocol are shear-stress dependent (Sinoway et al. 1986; Tinken et al. 2010; Green et al. 2017a). Such adaptations are unlikely to occur as a result of changes in the sympathetic tone (Sinoway et al. 1986). Changes in response of resistance or conduit arteries caliber can be, in part, attributed to adaptations in maximal blood flow or conductance due to training (Sinoway et al. 1986). However, specifically test, such as an ischaemic handgrip exercise (Naylor et al. 2005), was not applied, thus limiting the evaluation of possible arterial remodelling in the present study. Future studies investigating the effects of RMT on arterial remodelling are necessary. Indeed, it has been shown in an animal model that arterial remodeling is NO- and endothelium-dependent (Green et al. 2005). On this premise, despite the lack of FMD response after training in the present study, it is plausible that RMT provided a physiological stimulus (i.e., the exercise-induced increase in SR) that led not only to structural remodeling but also to an increase in NO bioavailability in young, healthy women. Further studies are needed to better clarify the physiological pathway related to the observed drop in cumulative SR.

Chapter 7-Study limitations

This dissertation comes with some known limitations. First of all, this study was not matched against groups of elderly and/or people presenting cardiovascular dysfunction. Considering the present results, and the previous literature on similar topic, it is likely that these populations could have obtained more beneficial due to the alter health condition at rest. Second, the prolongation of RMT and IQT training, as well as introducing assessments aimed to verified the follow up of the studies, they may have potentially disclosed other results and insights. Lastly, although samples of 12 and 10 participants, for RMT and IQT respectively, was higher enough to reach an adequate statistical power, the enrolment of a higher number of participants with different characteristics could have permitted to highlights some possible difference among the sex, age and physical exercise level.

Chapter 8- Conclusion

The positive effects observed in the present dissertation, in terms of increase in PLM and %FMD/SR values, after IQT and RMT respectively, may suggest that also small muscle exercises are able to raise peripheral BF in both involved and non-involved exercising area. Additionally, positive adaptation in the peripheral component of the vasomotor response could be detected after training when the peripheral blood flow stimulus was strong enough to trigger a series of events (Green et al. 2017b), finally leading to improvements in the vascular structure and function (Naylor et al. 2005).

Practical implications of this dissertation finding, small muscle exercise model, involving only a limited amount of muscles exercising, it could be used in disease populations presenting an exercise intolerance caused by an autonomic dysfunction, alteration in the cardiorespiratory

system, low mobility (such as elderly or athletes recovering after an injury) to improve, recover and/or maintain the endothelium functionality. Future studies are necessary to assess the effect of IQT and RMT in other type of disease population to fully elucidate the effect of them on the vasomotor response.

In conclusion: 1) eight weeks of RMT improved the vasomotor response mainly via an effect on the peripheral component, as suggested by the increase in the %FMD /SR due to a reduction in arterial shear stress during the FMD maneuver, rather than an effect on the global component of the vasomotor response, as highlighted by no differences detected in the HRV components; 2) eight weeks of IQT improved peripheral vasomotor response only in the lower limb vasculature directly involved in the training without affecting vascular functionality in the uninvolved upper limb, suggesting that IQT did not provide a sufficient stimulus to induce adaptations also in other districts' vasculature.

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