Dissertation

Retinal vascular function in health and disease

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Adam Saloň, BSc MSc

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Division of Physiology & Pathophysiology
Otto Loewi Research Center for Vascular Biology, Immunology, and Inflammation

under the supervision of
Assoz.-Prof. Priv.-Doz. Dr.med.univ. Nandu Goswami, PhD

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STATUTORY DECLARATION

I declare that this dissertation thesis is my own original work and I have fully acknowledged all individuals and organizations that have contributed to this thesis research. The acknowledgment has been made in the text to all other material used. In the present thesis and in all related publications the “Guidelines of the Medical University of Graz on Good Scientific Practice “were followed.

Graz, October 2023 e.h. Adam Saloň

DISCLOSURE

As this dissertation follows a cumulative thesis format, three first-author publications related to the dissertation topic are included within it. The three included manuscripts have been published in the following open-access journals:

(1) Saloň A, Steuber B, Neshev R, Schmid-Zalaudek K, De Boever P, Bergmann E, et al. **Vascular Responses following Light Therapy: A Pilot Study with Healthy Volunteers**. J Clin Med. 2023 Mar 13;12(6):2229.

(2) Saloň A, Neshev R, Teraž K, Šimunič B, Peskar M, Marušič U, et al. **A pilot study: Exploring the influence of COVID-19 on cardiovascular physiology and retinal microcirculation.** Microvasc Res. 2023 Nov 1;150:104588.

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LIST OF ABBREVIATIONS

ACs	Amacrine cells
AVR	Arteriovenous ratio
BMI	Body mass index
BP	Blood pressure
cGMP	Cyclic guanosine monophosphate
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
CRAE	Central Retinal Arteriolar Equivalent
CRVE	Central Retinal Venular Equivalent
CVDs	Cardiovascular diseases
DVC	Deep vascular complex
DVP	Deep vascular plexus
EDHF	Endothelium-derived hyperpolarizing factor
ET	Endothelin
HF	High frequency
HR	Heart rate
IOP	Intraocular pressure
IVP	Intermediate vascular plexus
LBNP	Lower body negative pressure
LDL	Low-density lipoprotein
LF	Low frequency
MCs	Müller cells
MLT	Maharishi light therapy
NO	Nitric oxide
NOS	Nitric oxid synthase
O·-2	Superoxide
ONOO-	Peroxynitrite
PDEs	Phosphodiesterases
PKGs	cGMP-dependent protein kinases
PWV	Pulse wave velocity
ROS	Reactive oxygen species
RPCs	Radial peripapillary capillaries

RPE	Retinal pigment epithelium
SOD	Superoxide dismutase
SVC	Superficial vascular complex
SVP	Superficial vascular plexus
TNF	Tumour necrosis factor

KURZFASSUNG

Hintergrund: Herz-Kreislauf-Erkrankungen stellen weltweit die häufigste Todesursache dar. Trotz jahrzehntelanger wissenschaftlicher Priorität hinsichtlich der Verbesserung der Prävention und Diagnose, sind kardiovaskuläre Erkrankungen nach wie vor weit verbreitet. Die kardiovaskuläre Forschung ist daher wichtig und notwendig, um die Folgen dieser Krankheiten zu verringern. Eine der wesentlichsten (Präventions-)maßnahmen ist eine regelmäßige, schnelle und unkomplizierte Gesundheitsuntersuchung, in die auch neueste Ansätze und Methoden einfließen sollten. Eine weltweit anerkannte und innovative Technik zur Untersuchung der kardiovaskulären Gesundheit ist das *Fundus*-Imaging bzw. die nicht-invasive Analyse der retinalen Mikrozirkulation. Dabei werden Eigenschaften und Veränderungen der retinalen Mikrozirkulation untersucht und analysiert, und als Indikator für kardiovaskulären Veränderungen in größeren Gefäßen herangezogen. Das regelmäßige individuelle Screening könnte somit einen wichtigen Beitrag zur Verringerung von Herz-Kreislauf-Erkrankungen und deren Folgen in der gesamten Bevölkerung darstellen. *Methodik*: Ziel der vorliegenden Dissertation war es, Veränderungen in Parametern der retinalen Mikrozirkulation an verschiedenen Stichproben von Gesunden und Patienten unterschiedlicher Krankheitsbilder zu untersuchen (siehe Abschnitt Ziele und Hypothesen). Zwölf Studien (sechs an gesunden Proband*innen und sechs an Patientenstichproben) wurden durchgeführt und werden zum Teil in der vorliegenden Dissertation abgebildet. Drei davon (Lichttherapie, COVID-19-Patient*innen und ‚Lower Body Negative Pressure'-LBNP) werden in der vorliegenden kumulativen Arbeit vorgestellt und analysiert. Zur Datenerfassung und Analyse der retinalen Mikrozirkulation wurden die Netzhautbildgebung (Fundus?) und die MONA Reva-Software (Version 2.1.1) verwendet. Alle Daten wurden mittels SPSS (IBM SPSS Statistics for Windows, Version 27.0., Armonk, NY, USA: IBM Corp) analysiert. *Ergebnisse*: Die Studie zur Lichttherapie ergab eine signifikante Abnahme der Parameter central retinal artery equivalent (CRAE: $p < 0{,}001$) und central retinal vein equivalent (CRVE) ($p = 0{,}002$) im Vergleich Lichttherapie zu Placebo, wobei die beobachteten Ergebnisse möglicherweise nur natürlich auftretende Schwankungen in der Mikrozirkulation abbilden und nicht die Wirkung der Lichttherapie. Eine signifikante Abnahme des CRVE (von 240,94 µm, SD: 16,05, auf 198,05 µm, SD: 17,36; $F(1,17) = 7.681$; $p = 0{,}013$) sowie ein Trend zur Abnahme des CRAE (von 138,87 µm, SD: 12,19, auf 136,77 µm, SD: 13,19; $F(1,17) = 3.810$; $p = 0.068$) wurden bei Patient*innen nach Erholung von einer Coronavirus-Erkrankung 2019 festgestellt. Darüber hinaus wurde in dieser Stichprobe ein signifikanter

Anstieg des systolischen Blutdrucks (von 142 mmHg, SD: 15, auf 150 mmHg, SD: 19, p = 0,041), eine Verringerung der Herzfrequenz (von 76 bpm, SD: 15, auf 69 bpm, SD: 11, p = 0,001) und ein Trend zu einem Anstieg der Pulswellengeschwindigkeit (von 11 m/s, SD: 3, auf 12 m/s, SD: 3, p = 0,095) festgestellt. Die Studie zum ‚Lower Body Negative Pressure' ergab hingegen keine signifikanten Veränderungen der retinalen Mikrozirkulation. Weder zwischen den einzelnen Zeitpunkten/Stufen des LBNP noch zwischen den Geschlechtern ergaben (−10, −20, −30, and −40 mmHg) sich signifikante Unterschiede. *Schlussfolgerung*: Zusammenfassend erlauben die vorliegenden Ergebnisse der drei Studien Einblicke in das komplexe Zusammenspiel zwischen verschiedenen Interventionen und physiologischen Parametern. Gleichzeitig unterstreichen sie den Bedarf an weiterer Forschung, um die komplexen Beziehungen zwischen Interventionen, physiologischen Reaktionen und deren klinische Bedeutung zu entschlüsseln. Diese Erkenntnisse können zu einer sachkundigeren Gesundheitspraxis oder zur Entwicklung gezielter Interventionen, die die kardiovaskuläre Gesundheit in verschiedenen Kontexten unterstützen und verbessern, beitragen.

Schlüsselwörter: Herz-Kreislauf-Gesundheit; Retina; Mikrozirkulation; Netzhautgefäße; Netzhautbildgebung; Lichttherapie; COVID 19; Unterdruck im Unterkörper

ABSTRACT

Background: Diseases of the cardiovascular system pose the main reason for death worldwide. The improvement of prevention, and diagnosis, of these diseases, was a scientific priority for decades, however, their prevalence remains abundant. The continuation of cardiovascular research is therefore important and necessary to decrease their consequences. One of the most important tools for achieving this goal is a regular, quick, and uncomplicated health examination, which part should include the most recent approaches and techniques. Fundus imaging or non-invasive analysis of the retinal microcirculation is a globally recognized and innovative examination of an individual's cardiovascular health. The technique evaluates the features and parameters of retinal microcirculation as a reflection of cardiovascular changes in larger vessels. Such regular individual screening may pose an important tool to reduce cardiovascular disease and its consequences in the entire population. *Methodology:* The dissertation aimed to investigate alterations in parameters of retinal microcirculation in various populations of healthy people as well as patients with different diseases (outlined in the Aims and Hypotheses section). The twelve studies/projects (six

healthy study samples and six different samples of patients) are partially included in this dissertation topic. The present cumulative dissertation includes and presents three of them (the population undergoing Light Therapy, the COVID-19 population, and the population undergoing Low Body Negative Pressure). The retinal imaging approach and MONA Reva software (version 2.1.1) were used to capture and analyse the retinal microcirculation over these studies/projects. All the data were analysed using SPSS (IBM SPSS Statistics for Windows, Version 27.0., Armonk, NY, USA: IBM Corp). *Results:* Significant reductions in the central retinal artery equivalent (CRAE) ($p < 0.001$) and central retinal vein equivalent (CRVE) ($p = 0.002$) parameters were observed when comparing light therapy and placebo conditions. However, the observed effect results may only reflect naturally occurring fluctuations in the microcirculation and not the effect of light therapy. A significantly narrower CRVE (from 240.94 µm, SD: 16.05, to 198.05 µm, SD: 17.36; $F(1,17) = 7.681$; $p = 0.013$) and a trend in the reduction of CRAE (from 138.87 µm, SD: 12.19, to 136.77 µm, SD: 13.19; $F(1,17) = 3.810$; $p = 0.068$) were found in patients recovering from Coronavirus disease 2019. Furthermore a significant increase in systolic blood pressure (from 142 mmHg, SD: 15, to 150 mmHg, SD: 19, $p = 0.041$), a reduction in heart rate (from 76 bpm, SD: 15, to 69 bpm, SD: 11, $p = 0.001$), and trend of increasing pulse wave velocity (from 11 m/s, SD: 3, to 12 m/s, SD: 3, $p = 0.095$) were recorded in this sample. The lower body negative pressure study did not show any significant changes in retinal microcirculation between the evaluated measurement points (−10, −20, −30, and −40 mmHg) or across the sexes of healthy individuals. *Conclusion*: In conclusion, the findings from these three studies collectively offer insights into the complex interplay between various interventions and physiological parameters. Overall, these studies underscore the need for further research to unravel the intricate relationships between interventions, physiological responses, and their clinical implications. These insights can lead to more informed healthcare practices as well as targeted interventions development to support and improve cardiovascular health in various contexts.

Key words: Cardiovascular health; retina; microcirculation; retinal vessels; retinal imaging; light therapy; COVID-19; lower body negative pressure

1 INTRODUCTION

Cardiovascular health includes the overall well-being of the blood vessels and heart, playing a crucial role in maintaining the optimal function of the body. Unfortunately, there are cardiovascular diseases (CVDs), which pose a significant danger to them. In fact, CVDs have become the main cause of mortality worldwide, thereby posing a big deal for global health. The World Health Organization reports that CVDs are the cause of 17.9 million deaths (approximately 32% of all recorded deaths across the globe) every year (1). CVDs manifest in various forms, targeting both the heart as well as blood vessels, such as coronary heart disease, stroke, and heart failure, which can have serious consequences on well-being and longevity. The big players in the development of CVDs are cardiovascular risk factors, which include increased BP, high blood cholesterol, smoking, diabetes... Many of these risk factors can be interconnected and mutually aggravate the progression of CVDs. The impact of CVDs on the population is therefore enormous, and addressing the prevalence represents a huge challenge for the global health system. Prevention aimed at reducing their incidence, morbidity, and mortality is the highest priority. Reduction in cardiovascular risk factors and diagnostic improvement to allow fast and effective treatments are two main strategies to face and fight to impact of CVDs. The simplest way is to focus on modifiable risk factors, like unhealthy lifestyles, poor diets and sedentarism, as well as the implementation of healthier behaviours and thereby mitigate the onset of CVDs. Encouraging people to perform regular physical activity, eat healthier, quit smoking, and manage conditions like diabetes can have a significant impact on maintaining a healthy cardiovascular system. On the other hand, reinforcing and supporting healthcare systems, such as professional equipment, personal capacity with adequate knowledge, finances, and infrastructure, have also a big and important impact on the characterisation of the CVDs risk in each person and therefore the setting of early and correct treatment. The complex screening programs and precise assessment can help with the identification of individuals with predispositions to CVDs and offer targeted interventions to minimize their risk. In addition, the early diagnosis of individuals already suffering from CVDs is very important to prevent future complications and premature deaths. Timely identification of these individuals allows fast initiation of appropriate treatment, including medication, lifestyle modifications, and other medical procedures, and thus improving the prognosis and quality of their lives.

1.1 Cardiovascular System: Function and Dynamics

The cardiovascular system principal function is the fast transportation of oxygen, nutrients, and other substances throughout the body. It relies on pressure differences created by pumps, resulting in fluid flow along a gradient. In the human cardiovascular system, the heart holds the contractile function and generates the pressure gradient. The cardiovascular system operates as a closed circulation (arteries, arterioles, capillaries, venules, and veins) within a specialized network of blood vessels. Blood vessels possess a complex structure of their walls, which include several layers, tunica intima, media, and adventitia. The tunica intima is lined by an endothelium that facilitates blood flow. The tunica media is made of elastic and muscular tissue, and it controls the diameter of the vessels. The tunica adventitia gives structural support. On one hand, the macrovascular network includes conduit arteries, veins, big arterioles, and venules, enabling blood's rapid transportation. On the other hand, the microvascular network is composed by small arteries, arterioles, intricate net of capillaries, and venules. Microcirculation, with its extensive surface area, plays a crucial role in peripheral vascular resistance and serves as the primary location for the exchange of gas and nutrients (2). Alterations in both function and structure of the microvascular system can have serious and negative impact on cardiovascular health, often preceding clinically recognized atherosclerosis or target organ damage (3). Systemic arteries, under high pressure, are the beginning of the bloodstream transporting blood and nutrients to organs. They contain more elastic tissue and less smooth muscle to adapt to stress. Arteries branch into arterioles, which transport blood to tissues and organs and primarily consist of smooth muscle. Capillaries, arising from arterioles, have thin walls made up of a single endothelial layer and facilitate the nutrients and waste exchange with neighbouring cells. The venous system, comprising venules and veins, receives blood from capillaries. Veins have thinner walls, less muscle tissue, and higher capacitance, enabling them to hold a significant portion of circulating blood. Valves and muscle contractions facilitate blood flow toward the heart. Overall, the peripheral vascular system and the human cardiovascular system collaborate to facilitate circulation by regulating blood flow and enabling nutrient exchange through an intricate blood vessels net. Therefore, understanding the dynamics between macro- and micro-vascular components is crucial for maintaining cardiovascular well-being.

1.2 Endothelium: Regulator of Microvascular Function and Health

The endothelium serves as a key regulator of microvascular function and structure, playing multiple roles to maintain vascular health. Firstly, it acts as a physical barrier, preventing leakage (4). Secondly, it functions as a metabolically active system to regulate tone of the vessels and homeostasis by secreting dilating, such as nitric oxide (NO), and constricting, such as endothelin-1, substances (5,6). NO, as one of the most important vasoactive agents, plays a significant role in microvascular health. Its strong vasodilative effects counteract the vasoconstrictors effect. The microvasculature counts on smooth muscle cells-mediated NO effect and following the vasodilatation It maintains microvascular function and prevents proliferation of smooth muscle cells as well as remodelling of vascular wall (7). Furthermore, NO as an anti-inflammatory agent inhibits adhesion and aggregation of platelets, and suppresses by endothelial cells and leukocytes-mediated expression of pro-inflammatory molecules predominantly via inhibiting the expression of NF-κB (8,9). Furthermore, the important feature of endothelium is its responsiveness to shear stress generated by the blood flow. The mechanoreceptors in the vessel lumen sense the shear stress to mediate adequate endothelial responses, where reactive oxygen species (ROS) are one of the key players (10). To prevent oxidative stress, the endothelium upregulates its antioxidant mechanisms when the shear stress is below physiological levels. While laminar blood flow, characterized by consistent shear stress, is vital for correct function of endothelium, as it increases the expression of anti-inflammatory and atheroprotective agents; turbulent blood flow, resulting in non-laminar shear stress, disrupts endothelial function and via reduction of NO production, and increase of inflammatory and atherogenic genes expression, promotes an endothelial proatherogenic phenotype (**Figure 1**) (11,12). Impaired vasodilatation mediated by endothelium occurs during increases in blood flow, especially in regions where turbulent flow is observed, such as vessel bifurcations. These areas are very sensitive to endothelium disruption and facilitate the atherosclerosis development (13).

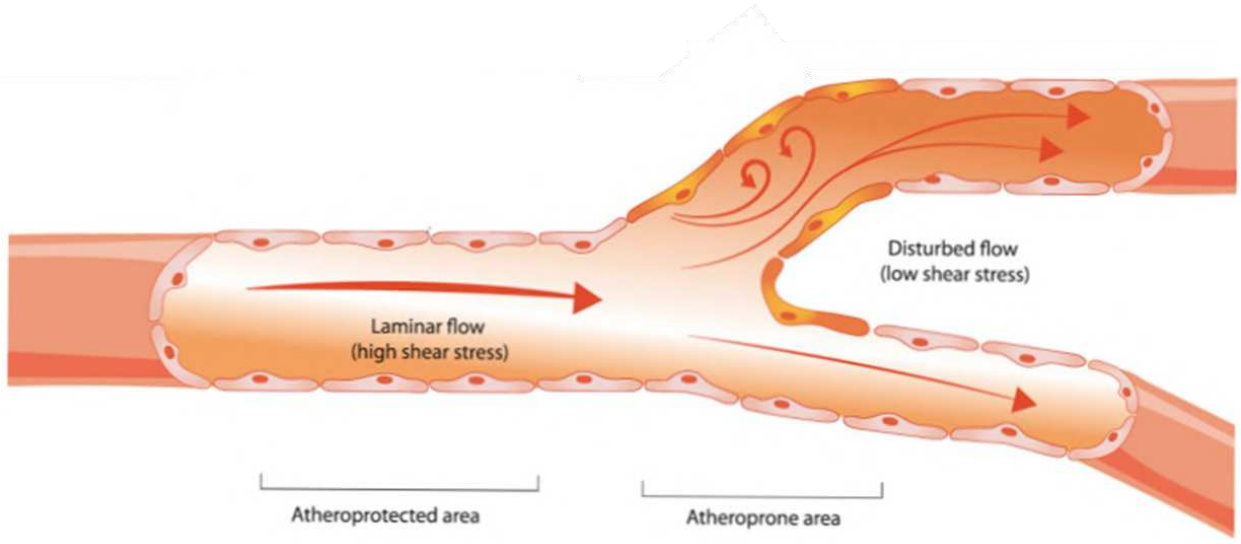

Figure 1: Shown shear stress in the area of bifurcation. The picture shows a reduction in shear stress in the area of bifurcation and therefore an induction of atherogenesis, as mentioned in the text. Adjusted from Bibli et al. (13)**.**

1.3 Regulation of blood flow

Blood flow regulation lies in the preservation constant flow of the blood over the perfusion pressure changes. Different organs display different degrees of autoregulation. There are 3 ways to autoregulation of blood flow.

Myogenic: constriction/dilation, as a response to blood pressure (BP) alterations, is regulated by smooth muscle cells, predominantly within arteries and arterioles. Stretching of these cells causes their depolarization, leading to contraction. When the BP goes up, the vessels constrict, and vice versa, when the BP goes down the vessels dilate.

Metabolic: the connection of the metabolic needs of the tissue with the blood flow. When tissue presents high metabolic rates, it releases vasodilative products, which in consequence will elevate the blood flow. As these metabolites fade away, they lead to vasoconstriction.

Endothelial: endothelial cells possess the ability of releasing several vasoactive substances as reaction to alterations in flow velocity and shear stress. The endothelium covering the retinal blood vessels can secrete mediators to modulate vascular tone and the diameter of the vessels according to the tissue request. Some of the main vasoactive factors produced by these cells are antagonistic-acting vasodilative NO and vasoconstrictive endothelin-1.

Disruptions in the vascular endothelium may lead to a dysregulation of vasoactive homeostasis, hence affecting the proper function of the circulation. It can be frequently seen in different pathologies, such as arteriosclerosis, hypercholesterolemia, or diabetes mellitus. Disturbance in autoregulation can lead to hypoxia which is followed by neovascularization, as we can see in diabetic retinopathy (14).

1.4 Vascular endothelial derived vasoactive agents

1.4.1 Nitric oxide (NO)

NO is the most relevant vasoactive agents made by the endothelium. It has an important role in vasodilation, inflammation, and oxidative stress. Disturbances in NO bioactivity may cause constriction of arteries, followed by endothelial dysfunction, which promotes vascular inflammation with the gradual creation of atherosclerotic plaque. Therefore, defects in the function of the endothelium often occur in CVDs. There are many conditions that can lead to impaired endothelial function by a reduction in NO bioactivity, the most common are hypertension, smoking, diabetes, and the ageing process.

Endothelial cells use L-arginine to synthesise NO, thanks to the nitric oxide synthase (NOS) enzyme, which may have three different isoforms (15). The ones involved with BP regulation are mainly endothelial NOS and neuronal NOS (15). Some important co-factors for the function of NOS are oxygen, NADPH, tetrahydrobiopterin, and flavin adenine nucleotides. The last isoform of NOS is inducible NOS, which is independent of Ca^{2+} and can be triggered by immune stimuli (15).

In a healthy endothelium, most of the NO is produced by endothelial NOS (16). The substances, such as acetylcholine, histamine, thrombin, serotonin, ADP, bradykinin, or norepinephrine are agonists of NO and can elevate its production and release from the endothelium. However, the shear stress in the vessels due to the blow flow is the main stimulant for NO synthesis. After being released, NO rapidly diffuses along the vasculature, with a half-life in blood estimated in the range of 0.05–1.8 ms (17). Erythrocytes play a crucial role in its transformation, reacting either with oxyhemoglobin to nitrate, with hemoglobin to nitrosylhemoglobin, or with the 93-cysteine residue of the β-subunit to S-nitrosohemoglobin (18).

Once NO is released it leads to the stimulation of soluble guanylyl cyclase and thus to the production of cyclic guanosine monophosphate (cGMP). This compound can interact with cGMP-dependent protein kinases (PKGs), cGMP-regulated ion channels, or cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs). Activation of PKGs in smooth muscle cells decreases the level of Ca^{2+} resulting in relaxation (19). On the other side of the endothelium, in the lumen of the vessels, signalling via PKGs leads to decreasing activation and adhesion of platelets (20). Additionally, NO inhibits the release of pro-inflammatory growth factors from the endothelium and from already bound platelets as well as the attraction of immune cells (21). Moreover, it also inhibits the release of the vasoconstrictors, endothelin-1, and norepinephrine (22).

1.4.1.1 Nitric Oxid in Inflammation

Vasodilatation is one of the typical signs of inflammation where NO as the main vasodilator plays a certain role. The inflammation process increases the level of pro-inflammatory mediators (bradykinin, histamine, and so on) in the circulation, which are responsible for the increased activity of NO. While NO released on the basolateral side causes cGMP- dependent smooth muscle cells relaxation, NO released on the luminal side is inactivated within seconds, predominantly by interaction with oxyhaemoglobin (22). Additionally, NO can be inactivated by interaction with superoxide ($O^{\cdot -2}$) to produce peroxynitrite (ONOO-). ONOO- can potentiate the vasodilatation effect of NO by triggering the relaxation mechanism in smooth muscle cells (23). On the contrary, ONOO- causes the oxidation of tetrahydrobiopterin and reduction of endothelial transport of L-arginine and therefore endothelial NOS uncoupling (24). It leads to the production of more $O^{\cdot -2}$ and subsequent ONOO- formation. Moreover ONOO- decreases antioxidant capacity by oxidizing the reduced glutathione and via nitration of superoxide dismutase (SOD), hence promoting the increase of ROS (25). SOD is the main inactivator of $O^{\cdot -2}$ by catalysing its dismutation to hydrogen peroxide (H_2O_2) and O_2. The increase of the $O^{\cdot -2}$ levels or decreased activity of SOD during inflammation leads to a reduction in NO bioavailability and promotion of inflammation.

ONOO- and $O^{\cdot -2}$ clearly support endothelial dysfunction, and apart from those mentioned above, they also have multiple other negative effects. ONOO- can inactivate prostacyclin synthase, which leads to the transformation of prostaglandin H2 to prostacyclin,

a known vasoprotective factor, hence promoting the formation of prostaglandins with vasoconstricting effects such as thromboxane A2 (24). Low-density lipoproteins (LDLs) can be oxidized by $O^{\cdot -2}$ as well as ONOO- giving as a result oxidized LDL. This compound can be scavenged by lectin-like oxidized low-density lipoprotein receptor-1, LOX-1, which ends up upregulating various adhesion molecules, chemokines, and proinflammatory agents as well as downregulating the eNOS, therefore supporting atherosclerotic plaque formation (26,27).

The endothelial cells activated by inflammation start expressing appropriate molecules, receptors, and cytokines, to attract immune cells. P-selectin on the luminal side of the endothelium can bind with selectins on the surface of leukocytes (e.g., L-selectin). This first touch of leukocytes with endothelium reduces the speed of their movement in the bloodstream and enables the creation of an additional bond between CD11 and CD18 molecules on leukocytes and endothelial integrins (22). The proper activity of NO decreases the levels of P-selectin via NO/soluble guanylate cyclase/cGMP signalling (28). Moreover, NO also hinders the amount of adhesion molecules in neutrophils (29) and therefore also reduces their aggregation and secretion followed by unfavourable overproduction of ROS. Disrupting NO's protective role leads to another typical sign of inflammation, leukocyte infiltration through the endothelium to a site of injury which is accompanied by increased production of pro-inflammatory mediators including ROS.

Another type of immune cell, important predominantly at the beginning of the inflammation is the mast cell. These cells are keepers of connective tissues and when recognize foreign antigens trigger the release of plenty of cytokines and signal factors such as serotonin, histamine, platelet-activating factor, PAF, tumour necrosis factor (TNF), metabolites of arachidonic acid and others, including NO. This reaction attracts other immune cells and coordinates the inflammatory response.

1.4.2 Thromboxane A2, Prostaglandin I2

Vascular function can be regulated both by prostacyclin, also known as prostaglandin I2, and thromboxane A2, which are metabolites of arachidonic acid. Their production is maintained due to the cyclooxygenase (COX)-1 expression, or upon inflammation COX-2 (30). Binding prostaglandin I2 to the appropriate receptor on platelets causes inhibition of their aggregation, and its interaction with receptors on smooth muscle cells triggers signaling via adenylate cyclase, cyclic adenosine monophosphate, and protein kinase A, which leads to

relaxation of the cells (30,31) same as NO does. Interestingly, when NO is present, silencing of prostaglandin I2 has no effect on vasodilatation (32), but with NO reduction, PGI2 compensates dilatation role (33). thromboxane A2 has opposite effects. It binds to its receptors on platelets and increases their aggregation (30). Binding to smooth muscle cells increases intracellular Ca^{2+} and leads to vasoconstriction. The equilibrium between these two antagonistic prostanoids seems essential for homeostasis in the vascular system.

1.4.3 Endothelin

Just as NO is considered the strongest vasodilator, endothelin (ET) is considered the strongest vasoconstrictor. ET has three isoforms, ET-1, ET-2, and ET-3, but only ET-1 is released and active in endothelial cells. Both the synthesis and release of ET-1 are induced by inflammatory markers like interleukins or TNF-a. On the other hand, its production can be reduced by NO and prostaglandin I2 (34). Additionally, shear stress may also reduce the expression of ET-1.

The interaction between ET-1 and its respective receptors (ET-A and ET-B2) on smooth muscle cells elevates Ca^{2+} inside the cells, which leads to vasoconstriction. On the other hand, endothelial cells possessing the ET-B1 receptor trigger vasodilation through the release of NO and prostaglandin I2 after ET-1/ET-B1 interaction (34). This opposite effect of ET-1 indicates that receptors and their expression rather than the ligands play the main role during the vasoconstriction of vessels. The typical sign of endothelial dysfunction is the downregulation of ET-B1 and at the same time upregulation of ET-B2 (35). The general effects of ET-1/ET-B interaction depend on an equilibrium of these 2 mechanisms. Moreover, the downregulation of ET-B can increase the level of unbound ET-1 causing ET-A-mediated vasoconstriction (36).

ET-1 can also have an important effect on several other events. It supports inflammation, smooth muscle cells proliferation in the vessels, activation of macrophages, neutrophil-vessel interaction, and increases the concentration of free radicals. All these mentioned activities of endothelin lead to the advancement of endothelial disorder.

1.4.4 Endothelium-derived hyperpolarizing factor

There is evidence that another factor named endothelium-derived hyperpolarizing factor (EDHF) fulfils the same function as NO. EDHF is released after the activation of endothelial cells by bradykinin or acetylcholine and works through the hyperpolarization of smooth muscle cells (37). As it was mentioned above, NO as well as prostaglandin I2, can cause vasodilation via the hyperpolarization of smooth muscle cells. The occurrence of this other factor – EDHF was confirmed when the vasodilatation effect was still preserved after the inhibition of NO and PGI2 (38). However, many signalling pathways can be involved in this hyperpolarization effect and the one that EDHF is responsible for remains unknown.

There are two main general mechanisms of EDHF action suggested (39). First, EDHF is a diffusible endothelial factor able to overcome internal elastic lamina and reach vascular smooth muscle cells to activate ion channels and therefore trigger hyperpolarization and relaxation of smooth muscle cells. The second suggested mechanism is maintained via intracellular contacts, which enable the passive transmission of endothelial hyperpolarization to the smooth muscle cells as an electrical signal. As a modulator of vascular tone, EDHF may have a crucial function in the maintenance of a healthy cardiovascular system as well as in the genesis of CVDs. However, there are still many uncertainties that need to be elucidated and future research could focus on clarifying them. Therefore, at this point, it may be more correct to talk about EDHF as the mechanism that can partially substitute the vasodilatation effect of NO and prostaglandin I2, rather than just about a specific endothelial factor.

1.4.5 Reactive Oxygen Species (ROS)

ROS are a product of oxygen metabolism and have an effect on a wide spectrum of signalling, immune response and the maintaining of the internal homeostasis of the organism (40). In normal physiological conditions their level is maintained low and stationary. On the other hand, ROS can pose danger for the body and cause irreversible damage of proteins, lipids or nucleic acids and prevent the cells from performing their physiological functions. Whether ROS will act as harmful or protective depends on balance between their production and removal, namely redox homeostasis, where the antioxidant mechanism plays an important role.

Interestingly, around 1–2% of the oxygen we inhale can cause oxygen radicals to form, and can potentially produce ROS (41). The oxidative phosphorylation in mitochondria and defensive mechanisms of the immune system suppy endogenous sources of ROS). It is therefore appropriate to consider that both, highly metabolically active cells, and ongoing inflammation result in an increase in ROS production. However, ROS can also be produced as a result of exposure to adverse external influences, such as radiation, pollutants or cigarette smoke (42).

In the body the main regulatory enzyme and antioxidant mechanism against oxidative stress are SODs. They trigger the reaction of $O^{\cdot -2}$ into molecular oxygen and hydrogen peroxide and therefore maintain the physiological concentration of superoxides (43,44). Moreover, NO can also react with $O^{\cdot -2}$ and form ONOO-, and thus compete with SODs. However, this reaction reduces the amount of available NO, which then promotes the development of dysfunctional endothelium (44).

Imbalance between free radical formation and the capability of cells to clear them initiates oxidative stress (40). Increased level of $O^{\cdot -2}$ reduces the NO available and elevates the synthesis of ONOO-. Furthermore, $O^{\cdot -2}$ has the potential to generate additional ROS. The role of NO can be partly covered by its agonists, but prolonged imbalance in redox homeostasis activates endothelium to production vasoconstrictors, and inflammation is initiated. Free radical clearance, incapability, disruption of vasoactivity, and the production of numerous chemotactic signals to attract immune cells lead to endothelial dysfunction (45). ROS can affect the function and regulation of a broad spectrum of signalling molecules, like phosphatases, protein kinases, or transcription factors. They activate protein kinase C, known as PKC, a protein that is included in multiple molecular pathways and one of them activates NOX (family NADPH oxidases) to increase the production of ROS (46,47). Additionally this signalling enhances the level of Ca^{2+}, which increases the effect of rho-associated coiled-coil protein kinase or ROCK, responsible for regulation of actin dynamics, and therefore to vascular contraction(48). PKC and ROCK are important players in vasoactivity and disruption of their function can be followed by vascular dysfunction.

Signalling between inflammation-attracted immune cells and their products with endothelium form a vicious circle of continuous inflammation, which can progress to endothelial dysfunction and later atherosclerosis-related complications. Therefore, ROS play a major role in many pathophysiological occasions, such as CVDs, inflammation, hereditary diseases, ageing, and many other diseases.

1.5 Endothelial Dysfunction and Microvascular Changes in Cardiovascular Diseases

Many factors associated with the cardiovascular system can disrupt the function of the endothelium and the structure and function of microvessels (49,50). These changes are key in the initiation and advancement of atherosclerosis and hypertension, often occurring before observable clinical manifestations of CVDs. Dysfunction of the endothelium by NO bioavailability reduction as well as endothelium-mediated vasodilatation impairment, play a major role in this process. Therefore, endothelial, and microvascular dysfunction may serve as the initial stage of hypertension and atherosclerosis development, significant contributors to diseases of the cardiovascular system.

Dysfunction of the endothelium plays an crucial role in the development of hypertension (51). In normal conditions, blood flow in vessels induces periodic stretch, eliciting a corresponding response of smooth muscle cells (52). However, when endothelium-mediated vasodilation is impaired, proper vasodilation does not occur, and the force exerted on the wall of the vessels increases. Consequently, the periodic stretching increases to pathological levels and compensatory mechanisms such as the proliferation of smooth muscle cells and elastin and collagen deposition to strengthen and stiffen the vessel wall. This leads to inward remodelling, vessel stiffening, increased vascular resistance, and elevated BP (52,53). In addition, the strengthening of the vessel wall leads to a reduction in its cyclical stretch properties, increases oxidative stress, reduces antioxidant defences, and promotes the expression of substances like Angiotensin-II and ET-1, further stimulating the proliferation of smooth muscle cells (54,55). This mutually reinforcing cycle of vascular changes amplifies endothelial dysfunction and hypertension (56). Hypertension can manifest in compromised blood flow to organs, small blood vessel ruptures, damage to target organs (mainly in the brain), heart failure, accelerated coronary vessels atherosclerosis, and left-ventricular hypertrophy. Furthermore, putting physical stress on the walls of the arteries also leads to atherosclerosis acceleration and contributes to plaques rupture.

Oxidative stress as well as dysfunction of the endothelium are important in the origin and development of atherosclerosis (54). Disruptions in endothelium-mediated vasodilation occur before the formation of the atherosclerotic plaques, therefore suggesting, that microvascular changes underly the disease before clinically detectable macrovascular changes (4). As mentioned before, the reduced availability of NO leads to increased activity of NF-κB,

thereby pro-inflammatory molecules like cytokines and adhesion molecules that support extravasation are elevated. In addition, reduction in NO is hand by hand with inactivation of fibrinolytic factors and activation platelets, that promote thrombus formation (4). As mentioned in the section *Vascular endothelial-derived vasoactive agents*, ROS can oxidize LDL and cause an upregulation of various adhesion molecules, chemokines, and proinflammatory agents to further exacerbate the inflammatory process (26). Oxidized LDL promotes the extravasation of monocytes, thereby support their differentiation into macrophages. Extravasation and increased amount of macrophages trigger the fatty streaks formation and the release of pro-inflammatory cytokines (26,57,58). The gradual leukocyte and mast cell accumulation in the subendothelial space triggers communication between immune cells and intensifies the pro-inflammatory status (59).

Smooth muscle cells are attracted and invade the intima-media, where they run the production of extracellular matrix proteins to cover the lesion by creating a fibrous cap (57). Within the fibrous cap, lipid-rich pools known as necrotic cores develop from dying foam cells (60). The atherosclerotic plaque stability depends on the fibrous cap thickness (61). The stable plaques with intact fibrous caps can cause flow-limiting stenosis, resulting in ischemia of the tissue and stable angina. Vulnerable plaques in which the fibrous caps are thin and prone to rupture, which is often triggered by hypertension. Rupture of the plaque exposes the core to coagulation proteins in circulation, resulting in thrombosis, partial or complete occlusion of the artery lumen, and in the last stages into acute coronary syndromes or stroke (59,61).

1.6 A Brief Overview of Cardiovascular Disease Risk Factors

CVDs are influenced by various risk factors that contribute to increased morbidity and mortality. Typical risk factors such as aging, obesity, hypertension, diabetes, and atherosclerosis play significant roles in the development of CVDs.

Aging often leads to high BP, reducing the elasticity of arteries and reduces blood and oxygen flow. High BP, particularly in the pre-hypertensive range, is strongly associated with increased occurring of CVDs, stroke, and myocardial infarction (62). Long-term hypertension not only contributes to the development of CVDs but also increases the risk of mortality (63,64). Obesity poses another risk factor of CVDs, often co-occurring with hypertension and/or diabetes. The measure of the obesity through evaluation of body mass index (BMI)

shows higher hazard ratios for coronary heart disease and stroke (65). High level of blood cholesterol is another risk factor for CVDs. Elevated cholesterol, particularly LDL cholesterol, leads to the formation of fatty plaques in arterial walls, resulting in atherosclerosis and narrowed arteries. This limitation of blood flow contributes to the development of hypertension, further increasing the risk of CVD (66). In addition, smoking negatively impacts cardiovascular health. It increases level of LDL cholesterol and triglycerides, while decreasing levels of high-density lipoprotein (HDL), thereby promoting the development of CVDs (67). Diabetes is characterized by high blood sugar levels, that can damage blood vessels over time. Increased levels of sugar in the blood lead to higher levels of oxygen radicals and reduced bioavailability of NO, which can contribute to the narrowing and decreased elasticity of blood vessels (68,69). The individuals with type 2 diabetes showing more than twice the risk of coronary heart disease (70). Additionally, diabetics are more prone to developing high BP or obesity, which further increasing the risk of CVDs. These risk factors collectively contribute to the development and progression of CVDs. Managing and addressing these factors through lifestyle modifications, proper medical care, and targeted interventions can significantly reduce the incidence and impact of CVDs.

1.7 Eye: Key Components and Functions

The eyes are the primary organs responsible for photoreception. The eye itself can be divided into three main layers: the outermost sclera, followed by the choroid, and finally the retina. While the sclera protects this apparatus and the choroid nourishes it, the retina is made up of sensory cells, rods, and cones (71,72). The cornea, formed by the sclera, allows light to enter the eye, while the iris and ciliary bodies, derived from the choroid, contain muscles and glands (72). Located in front of the lens is the aqueous humour, and behind the lens lies the vitreous humour, which makes up about 80% of the volume of the eye (73). Extending from the corneal epithelium is the conjunctiva, which connects the eyeball to the eyelid and provides protection and nourishment to the cornea (72). The eyelids play a crucial role in safeguarding and cleansing the eyeball. When light reaches the retina, pigments such as rhodopsin (in rods) or iodopsin (in cones) undergo conformational changes, triggering nerve impulses that are transmitted to the visual processing system (73).

1.7.1 Anatomy and Structure of the Eye

As mentioned above, the eye consists of three coats, which enclose the AH, lens, and vitreous body. The sclera and cornea make up the outermost coat of the eye. The middle coat, named the uvea, is responsible for blood supply and consists of the choroid, the ciliary body, and the iris. Finally, the innermost coat is the retina, which sits on the choroid. These, along with many other parts of the eye, are described in more detail in the following paragraph (**Figure 2**).

The eyes lie in protective cavities of the skull, named bony orbits. Six extraocular muscles of the eye are connected to the sclera and control eye movement up and down, side to side, and rotation. The sclera is an outer layer of the eye that covers almost the whole eyeball surface. A clear membrane conjunctiva covers the eye surface and the inner surface of the eyelids. It protects the eye from bacteria and foreign materials and contains blood vessels, which are visible against the white background of the sclera.

The front of the eye is lubricated by tears/tear film composed of three layers. The conjunctiva makes the mucous layer, the lacrimal gland makes the watery layer, and the oil part of the tear film is made by the Meibomian gland. The tear duct drains tears from the eye.

Light enters the eye through the clear, convex-shaped front part of the eye, the cornea. Between the cornea and the forward surfaces of the iris and lens is the anterior chamber. The posterior chamber is a smaller area located between the rear surface of the iris and the ciliary body and lens. Both chambers are filled with an aqueous humour and are connected through the pupil. The eye produces aqueous humour to nourish and maintain eye pressure. Drainage of aqueous humour in the drainage angle helps to maintain a constant eye pressure. High intraocular pressure (IOP) can be a sign of glaucoma or other eye disorders and can damage vision. Behind the anterior chamber is the pupil, which is the centre of the iris and allows light to enter the eye. The iris controls the size of the pupil. The iris is surrounded by the ciliary body, a ring of tissue that connects it to the choroid coat. The choroid is the posterior part of the uvea, sandwiched between the sclera and the retina, and consists of the iris and ciliary body. At the back of the pupil is the lens, attached to the eyewall and surrounded by the lens capsule. The lens modulates its own shape to focus beams of light on the back of the eye. Focusing light by the cornea (70%) and the lens (30%) allows us to see. In the middle of the eye, between the lens and the backside of the eye, is the vitreous cavity filled with vitreous humour. The retina is a tissue sensitive to light and covers the back wall inside the eye, which

sends impulses of electric signals in the form of action potentials to the brain, using the optic nerve.

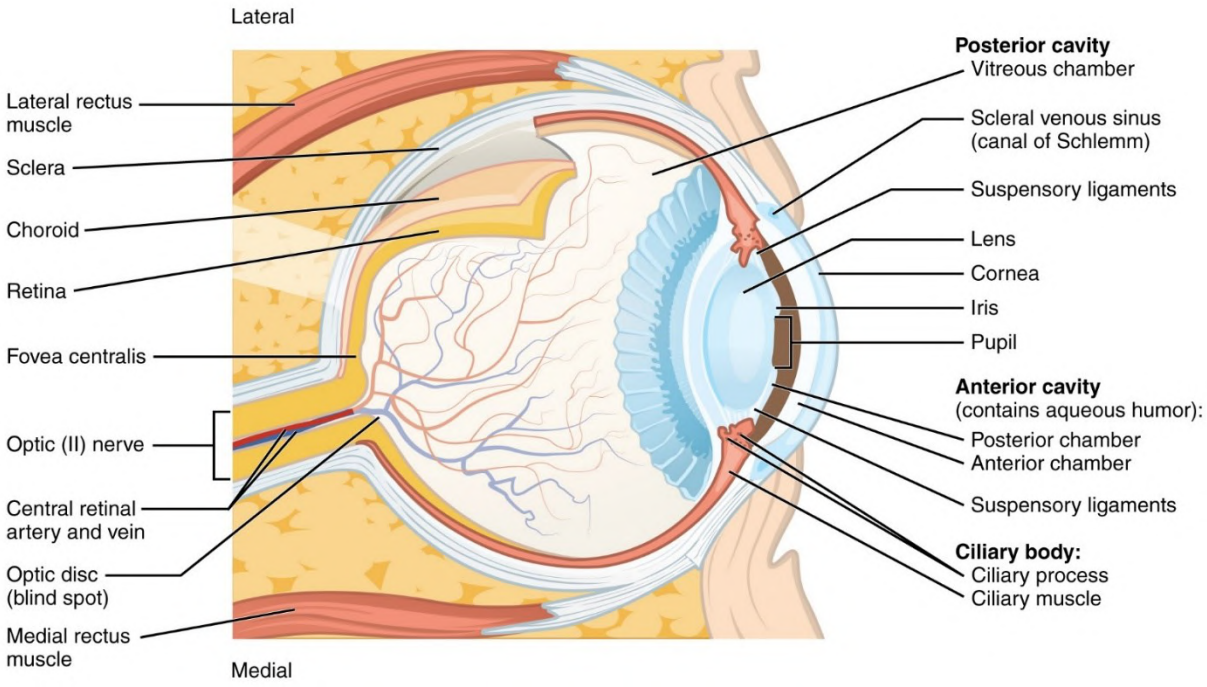

Figure 2: A simplified image of eyes anatomy displaying most of the structures mentioned in the text above. The image was taken from www.opto.ca *(74)*.

1.7.2 Anatomy of retina

The number of cones and rods in the human retina is estimated at 7,000,000, respectively from 75,000,000 to 150,000,000. To reach the rods and cones, lying almost the innermost, the light must pass through all rest layers of the retina. Subsequently, the light is absorbed by the **retinal pigment epithelium** or **choroid**. In the **fovea** region are all retinal layers above the photosensitive cells diverted to sides. It facilitates light access to the photosensitive region and ensures sharp and central vision. In the center of the **fovea**, on the temporal side of the **optic disc,** is the region named **fovea centralis**. Depression in the middle of **fovea centralis** is the **foveal pit**. It is a specialized part of the retina with a diameter of around 200 microns. In this area, the cones are very narrow, long, and tightly packed in contrast to the thicker cones more peripherally. Around the **fovea** is the **parafovea region**, 1,250 microns from the centre, and here is the highest density of rods. The six layers of ganglion cells in the **parafovea** (75), make this area the thickest part of the retina. Around the

parafovea is the **perifovea area**, the most outer edge of **fovea** and it is 2,750 microns from its centre. The density of cones is reduced here to compare to the centre.

The area in the retina named the **optic disc** or **blind spot** is the place on the nasal side of the retina through which the fibres of the **optic nerve** leave as well as nourishing and draining retinal vessels enter and leave the eye.

The outermost edge of the retina is the **ora Serrata**. It connects the retina and the **ciliary body** and represents the transition from a non-photosensitive region of the ciliary body to a multi-layer photosensitive region of the retina.

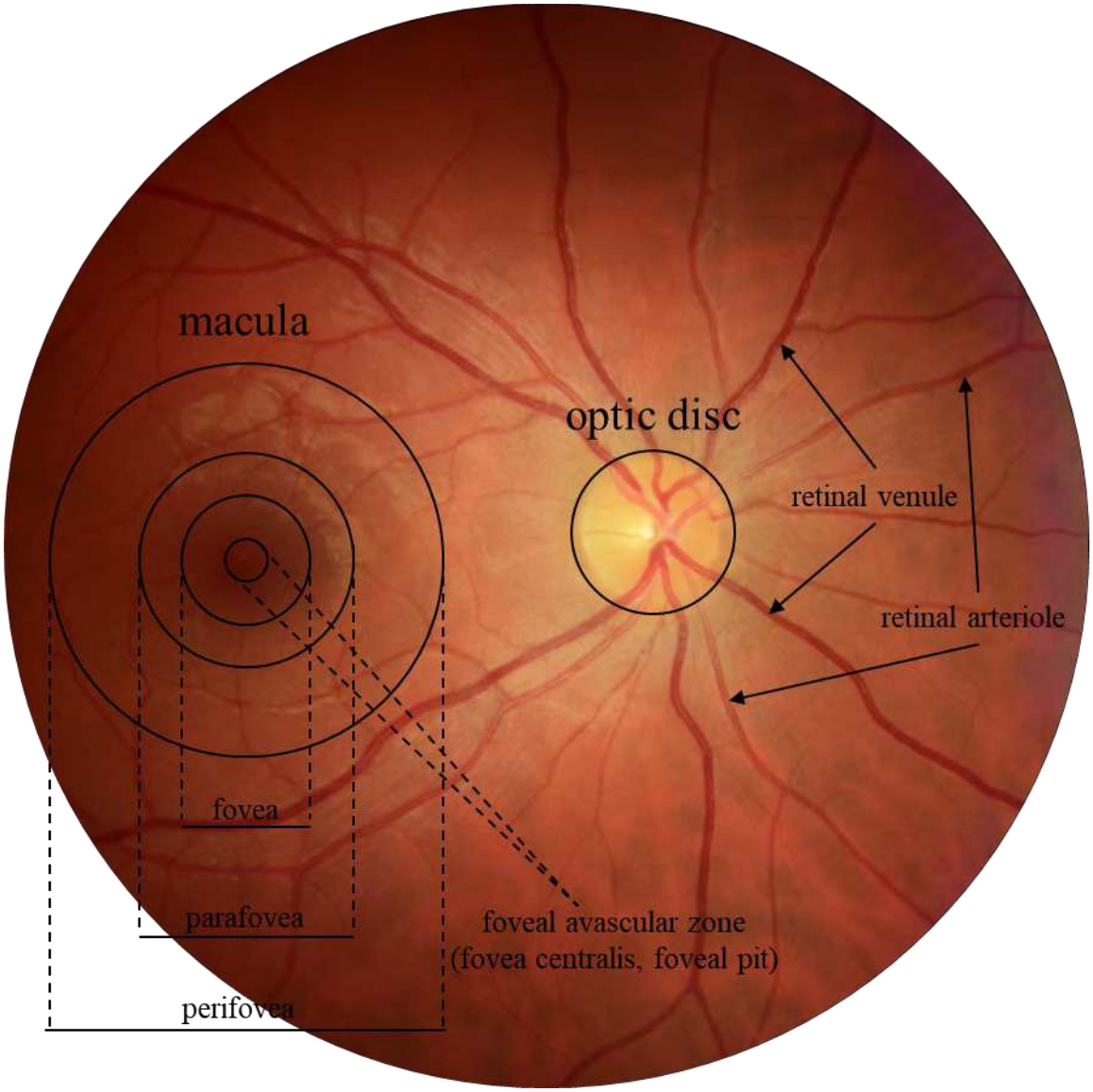

Figure 3: Retinal fundus image of the human eye, with highlighted key anatomical parts such as the macula, perifovea, parafovea, fovea, optic disc, fovea centralis, and foveal pit. The image shows also randomly picked two retinal arterioles and venules.

1.8 Overview of Ocular Blood Flow and Circulation

Absolute human blood flow of human eyes is estimated to be around 1 ml/min (76). The arterial flow to the eye is assured by ophthalmic artery branches, what is derived from the internal carotid artery. The two main branches involve: 1.) the central retinal artery; and 2.) the posterior ciliary artery.

We distinguish two types of circulation that supply the eye (choroidal and retinal). The outer retina including the anterior part of the eye is nourished via choroidal circulation represented by the anterior ciliary artery as well as long and short posterior ciliary arteries. The posterior eye (retina) is nourished in both ways of the eye's circulation. Outer one-third is nourished through choroidal circulation by posterior ciliary arteries, specifically by choriocapillaris. The inner two-thirds are supplied through retinal circulation via the central retinal artery and its branches.

The eye is nourished by one ophthalmic artery and drained via two ophthalmic veins. The drainage progresses via vortex veins and the central retinal vein which merge with superior and inferior ophthalmic veins and continue into the sinus cavernosus and subsequently to the pterygoid venous plexus, and the facial vein.

1.8.1 Fundamentals of Retinal Circulation and Vascular Dynamics

The retina is highly metabolically active tissue. The outer retina possesses the most metabolically active tissue in the body. The oxygen consumption is faster and in more amounts here than in the brain (77). Most of the retinal blood is carried by choroidal circulation and only around 10 % remains in the retinal circulation (14,78). While the flow of the blood in choroidal circulation is higher than the flow in retinal circulation (2000 ml/min/100 g tissue versus 60 ml/ min/100 g tissue), there is low choriocapillaris-mediated oxygen extraction (79). The high flow and amount of blood in choroidal circulation can be explained by the low level of oxygen extraction. The high flow provides high tension of oxygen and enhances its diffusion through Bruch's membrane and the RPE to mitochondria in the inner segment of the photoreceptor layer, an extremely metabolically active part of the

retina. This phenomenon also suggests that choroidal circulation may have additional functions, for example, it can work as a volume buffer, have a thermoregulation function, and play a role in aqueous humour formation(14).

Along with pathophysiological conditions, natural aging processes cause gradual structural and associated functional changes in the retinal circulation. While from a pathophysiological perspective it is mostly about vessel changes through inflammation, the gradual aging process causes cellularity loss in the peripheral capillaries, capillaries reduction around the fovea, or choroid size reduction. Both cases can culminate in arteriosclerotic-related changes such as vessel wall stiffening and hyalinization, muscular layer hyperplasia, and fibrinoid-related vessel wall necrosis. These changes can reduce oxygen and nutrients supply, disrupt the nourishment of retinal photoreceptors, and result in deteriorating sight.

1.8.2 Oxygen distribution in retina

The oxygen distribution varies from tissue to tissue. Tissue nourishment requirements are predominantly a reflection of its metabolic activity. The choroid has the highest oxygen pressure in the eye. On the other hand, the lowest is due to high oxygen demand by myriads of mitochondria in inner segments of photoreceptors. This high oxygen consumption, together with the avascularity of the outer retina, can pose a serious risk of hypoxia even with small circulation disturbances. Moreover, as mentioned earlier, the choroidal circulation has a low rate of oxygen extraction and due to the absence of autoregulation, the risk of oxygen distribution is amplified, especially in dark adaptation when inner segments of photoreceptors have a higher oxygen demand for ATP production (80).

The choroid supplies around 90% of the oxygen to maintain the proper function of the photoreceptors in the dark, and all the oxygen during adaptation to light (81). To provide adequate oxygenation, the oxygen usage of the outer retina can be increased. It is highly likely that to provide adequate oxygenation, the capillaries from deep vascular plexus (DVP) expand to the inner layer of photoreceptors and provide support. Hypoxia in these locations can lead to the development of age-related macular degeneration or diabetic retinopathy (82).

1.8.3 Blood Flow Regulation in Choroidal and Retinal Circulations

Choroidal circulation is regulated predominantly by the autonomic nervous system. On the other side, retinal circulation has no autonomic innervation, and it utilizes autoregulatory mechanisms. Autoregulation in both systems operates within specific pressure limits, causing blood vessels to dilate or constrict within their maximum capacity.

In the choroid, autonomic innervation, specifically sympathetic vasoconstriction and parasympathetic vasodilation, plays a crucial role in regulating blood flow (83). However, studies have shown that the choroid also exhibits some level of autoregulation (14,84). As pointed out above, the blood flow elevation in the choroid is believed to contribute to temperature stabilization and protection of the retina against thermal damage (14,84). Thus, proper regulation is necessary to prevent hypoxia, thermal stress, or other potential damages to the retina.

In the retinal microcirculation, the structure closely resembles that of general vessels. Arteries of the retina comprises of a well-developed tunica media consisting of smooth muscle cells lined in five to seven layers (85). As arteries divide into precapillary arterioles, the number of smooth muscle cells and layers gradually decreases. Unlike the autonomic regulation observed in choroid circulation or other arteriolar systems, autoregulation becomes the primary regulatory system in the retinal microcirculation, influencing the diameter and shape of arterioles (Yu et al., 2016). In contrast to arteries, the wall of the retinal venules is thin structured of one layer of endothelial cells supplemented by only a few smooth muscle cells (86,87).

By combining autonomic and autoregulatory mechanisms, the choroidal and retinal circulations maintain their unique characteristics within the overall vascular system, ensuring proper blood flow and functionality of the retina.

1.8.4 Choroidal circulation

Most of the blood flow in eyes is delivered by the choroidal circulation. It supplies the outer layers of retina, such as the layer of photoreceptors or RPE layer. This part of the retina has high metabolic activity and healthy choroid vasculature is fundamental for the right function of the eye. Additionally, the choroid is responsible for absorption of the light, thermoregulation, and blood flow-mediated vasomotor modulation of IOP (14). Choroid

circulation is also important in aqueous humour drainage from the anterior chamber. Disruption in this part of the circulation can deteriorate the function or cause death of both RPE and photoreceptors. The choroid gains blood from anterior and posterior ciliary arteries branches as well as ophthalmic artery branches. Generally, 6-12 short posterior ciliary arteries enter the sclera at the optic disc and subsequently branch into the network of arterioles and forming the choroidal dense outer layer. These perpendicular and terminal arterioles supply choroidal capillaries creating the vascular bed. Individuals with a cilioretinal artery have an additional or alternative blood supply to the retina. Cilioretinal arteries are contained the system of posterior ciliary arteries (88). They can grow either from one of the posterior ciliary arteries or directly from the choroid (88).

The previous research is not very consistent on the occurrence of cilioretinal artery. It is reported to be between 15% to 50% of population (79). It typically comes from the optic disc temporal side to reach the retina and nourish the macula (79). A small part of this circulation also supplies the anterior ciliary arteries.

1.8.4.1 Drainage (choroidal circulation)

Choroidal blood is drained via vortex veins. The lobules of choroidal capillaries lead blood to venules converging to bigger venules of the outer conduit layer. These subsequently continue in flow into the vortex veins. Each of the four quadrants is drained by one or two vortex veins which penetrate the sclera at the equator. They coalesce to either superior or inferior ophthalmic vein and then flow to the sinus cavernous.

1.8.5 Retinal circulation

The retinal circulation is primarily responsible for the nourishing and draining of approximately 2/3 of the inner retina. However, because of the huge demand for oxygen, for instance during dark adaptation, oxygen delivery can expand up to the inner photoreceptors. Retinal circulation is involved in supplying exclusively the retina and is made of the central retinal artery as well as branches of the central retinal artery. It infiltrates the eye at the head of the optic nerve, then continues to penetrate through the sclera, and branches into superior and inferior papillary arteries. These branches continue to divide into nasal and temporal quadratic branches and spread out to nourish the layers of the inner retina. Their obstructions

leading to limitation or cessation of circulation can very quickly lead to serious visual disturbances and even blindness.

The vessels of retinal circulation are spread in two vascular complexes. The superficial vascular complex (SVC) spreads through the nerve fiber layer, the ganglion layer, and partially in the inner plexiform layer (89). The deep vascular complex (DVC) maintains nourishment of the inner plexiform, inner nuclear, and outer plexiform layers, with Henle's fiber layer within it (89). Every one of these vascular complexes covers 2 vascular plexuses. From inner to outer, while the SVC covers radial peripapillary capillaries (RPCs) and superficial vascular plexus (SVP), the DVC encompasses the intermediate vascular plexus (IVP) as well as DVP (89,90). The diameter and density of capillaries are significantly different between the four plexuses (90). The vascular connections between the plexuses have been shown by Stephane Fouquet et al. (91) (**Figure 4**). Most of the arterial flow is in the SVP. The SVP divides to RPCs on the one side and to the IVP with its serial connection to the DVP on the other. The flow of the blood then passes via RPCs and continues to the IVP or DVP. To sum up, the retinal capillary flow is comprised of serial flow from SVP to DVP and parallel-connected RPCs (**Figure 4**).

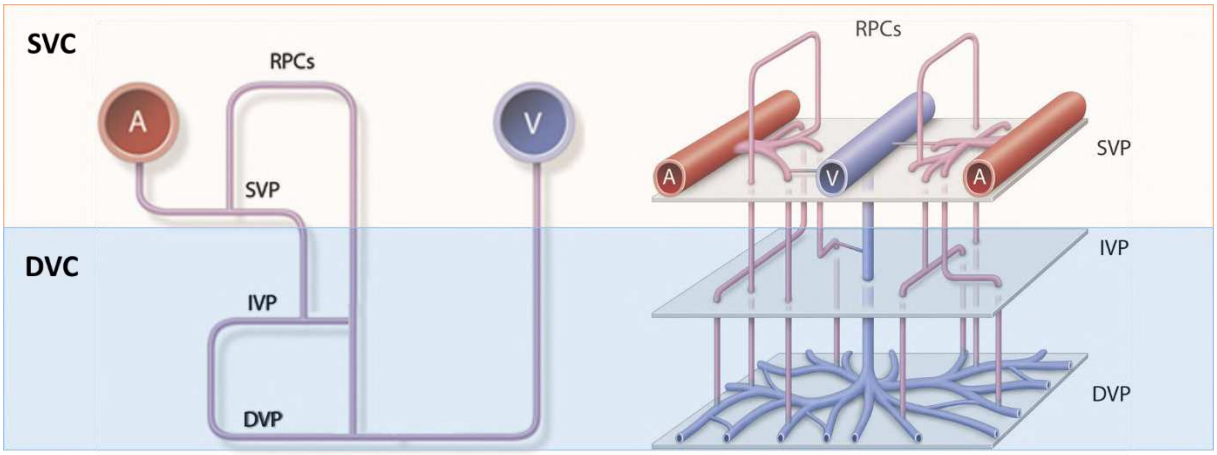

Figure 4: Proposed retinal microcirculation models in pig retinas. 2D and 3D presentation of proposed models of retinal microcirculation with their interconnections. The model was designed by Fouquet and colleagues; the image is taken and adjusted from their publication *(91)*. Superficial vascular complex (SVC), deep vascular complex (DVC), radial peripapillary capillaries (RPCs), superficial vascular plexus (SVP), intermediate vascular plexus (IVP), and deep vascular plexus (DVP).

1.8.5.1 Radial peripapillary capillaries (RPC)

The RPC makes up the outermost plexus layer. It is in the nerve fiber layer and ganglion cell layer, slightly beneath the internal limiting membrane. Its typical feature is that it contains the large retinal arteries. The blood vessels of RPCs extend into the peripapillary zone, and span 4-5mm from the optic disc (92). They can be visualized with a retinal camera or ophthalmoscope.

1.8.5.2 Superficial vascular plexus (SVC)

The SPV is created by the retinal arterioles of both RPCs on one, and IPV and DPV on the other side. It is the sole plexus nourished by branches of precapillary arterioles and drained by branches of postcapillary venules (90). Subsequently, arteries of SVP move the blood to other parts of retinal circulation and form an interconnecting network between all retinal plexuses. The smallest representation of SVP is on the periphery of the retina (89).

1.8.5.3 Intermediate vascular plexus (IVP)

The IVP arises primarily from SVP but can be supplied also by blood from RPCs. It continues to DVP. IVP lies partially in the inner plexiform layer with extensions into the inner nuclear layer (91,93).

1.8.5.4 Deep vascular plexus (DVP)

It is the deepest vascular plexus of retinal circulation extending to the outer plexiform layer. It is the part of the circulation that, when needed, is probably responsible for supplying oxygen up to the internal photoreceptors. Together with the IVP, they have the largest representation in the peripheral zone of the retina (89).

1.8.5.5 Drainage (retinal circulation)

The drainage of retinal circulation is provided by venules and veins of the retina. Afterward these coalesce into the central retinal vein. The central retinal vein exits the eye, centripetal, alongside the central retinal artery and the optic nerve in the optic disc location.

After exiting the ocular region, it flows together with the vortex veins. Both then connect to the superior ophthalmic vein, entering the sinus cavernous.

1.8.6 Retinal Microvasculature Resembles Cerebral and Coronary Systems

Embryological development of both the eye and brain exhibit similar vascularization processes, thereby pointing to the anatomical likeness between microcirculation in retina and brain (94,95). The retina, as well as the brain, typically has dense capillary networks, with endothelial cells creating strict barriers to maintain neuronal integrity and control the transfer of molecules (96,97).

In organs with high metabolic demands like the retina, brain, and coronary system, autoregulation plays a vital role in controlling blood flow (98,99). Myogenic autoregulation, involving smooth muscle contraction in response to increased pressure, helps maintain constant blood flow. Metabolic autoregulation adjusts blood flow through vasodilation or vasoconstriction based on tissue metabolic activity (98,99). The key player to regulate vascular tone in these circulation systems is the constant production of NO that ensures adequate blood supply and reduces perfusion pressure.

These microvasculature systems (retinal, cerebral, and coronary) are designed to minimize shear stress and BP (100,101). However, various circumstances can influence the vasculature and alter the structure of the vessel wall, as well as function in these systems (102). The fundus photography technique visualizes microvasculature in the retina in vivo and provides insights into the interconnectedness with cerebral and coronary circulation. This opens diagnostic possibilities for conditions affecting these microvascular systems.

1.9 Analysing Fundus Images and Detecting Retinal Abnormalities

The initial step in analysing fundus images involves detecting retinal landmarks including the optic disc location, fovea, and vasculature (103). Algorithms utilize techniques like principal component analysis and geometrical parametric models to distinguish the structure of an optic disc from other structures (104). Retinal blood vessels are detected by analysing their distinct distribution against the intensity of the retinal background (105). Common approaches include matched filters, vessel tracking algorithms, and neural networks

for automated vessel identification (106).

Calibration plays a crucial role in computer-assisted programs to determine the dimensions of fundus features. Previous studies have established a standard optic disc diameter in microns ranging from 1,800 to 1,900, while the optic disc to the macula length is around 2.5 times the diameter of the disc (107). This widely recognized standard is an internal reference to smooth out the effects of camera zoom. In addition to enabling the measurement of single vessel widths, automated vessel detection algorithms, as discussed earlier, offer limited information due to factors like heart rate (HR) or autonomic nerve stimulation (108,109). To overcome this limitation, retinal vascular features (such as Central Retinal Arteriolar Equivalent [CRAE] and Central Retinal Venular Equivalent [CRVE]) was introduced comprehensive measurement of the ratio between retinal arterioles and venules, namely the arteriovenous ratio (AVR), providing valuable insights into disease status. AVR calculations, based on formulas by Parr and Hubbard, involve estimating arteriolar and venular vessels within a predefined zone located between concentric rings when one of them is located at 0.5 and the second one is 1 optic disc diameter from the disc margin (109,110). Parr et al. and Hubart et al. used all arteriolar/venular widths to calculate two main retinal microcirculation parameters: CRAE and CRVE (109,110).

The connection between AVR reduction and high BP or diseases of the cardiovascular system indicated a reduction in the diameters of retinal arterioles, as it was expected that the arteries are influenced more than venules in relation to CVDs (111). It was discovered later that venules and arterioles respond differently to pathological conditions, leading to separate calculations for CRAE and CRVE. Moreover, up to that date, the count of vessels to be used for the AVR calculation posed evident limitations. However, Knudtson et al. revised the AVR formulas to reach the point that today only the six largest vessels from both arterioles as well as venules, are used for calculation CRAE, respective CRVE (112).

1.9.1 Exploring Additional Parameters in Retinal Microvasculature Quantification

While the present dissertation thesis clearly investigates the relationship between retinal vessel diameters and their modulation by multiple health/disease conditions, the following paragraph will briefly outline the additional valuable parameters that can be scrutinized in retinal vasculature.

Besides the analysis of the diameters, the retinal imaging technique enables the analysis of the other parameters containing valuable prognostic information.

One of them is the bifurcation angle, which, as the name says, is the angle formed at the vascular junction between two daughter vessels. It indicates the pattern of vascular branching and the vascular network density. This angle has a certain, optimal value. The optimal bifurcation angle value depends on the surface area, volume, drag, or repulsion and how symmetric the daughter vessels are (113–115). A reduction in this value is associated with a reduction in vascular network density and correlates with hypertension and aging (102,116).

The second additional parameter is vascular tortuosity. Vascular tortuosity refers to the ratio between the actual distance of a blood vessel from point A to B and the shortest distance between these points made by a straight line. It is the degree of twisting or curving in the path of retinal blood vessels, often indicating changes in vascular structure associated with various health conditions. Healthy blood vessels typically exhibit none or minimal curvature, respective straightness. Nevertheless, multiple pathological conditions can lead to collagen reduction and elastin elevation in the vascular wall, with result of an increase in vascular tortuosity. An increase in retinal arteriolar tortuosity observed around the optic disc is an early indication of disease (105,117).

The next vascular parameter that can be measured and evaluated via retinal imaging technique is the length-to-diameter ratio. The length: diameter ratio is calculated by measuring the distance from the midpoint of a specific vascular bifurcation to the midpoint of the previous bifurcation. Then is this measurement expressed by a ratio to the diameter of the parent vessel at the bifurcation area. Retinal arterioles that are longer and/or thinner can attenuate greater pressure. This ratio enhances the prognostic value of retinal imaging, particularly by assessing retinal arteriolar characteristics. It acts as an indicator of retinal arteriolar narrowing which is confirmed by its increase during hypertension (118,119).

The above-discussed patterns of retinal vasculature are based on either the diameter or shape of the vessels. The comprehensive structure of retinal microvasculature can be further investigated via fractal analysis. The fractal analysis evaluates the fractal dimension, representing how precisely the network of retinal vasculature occupies the 2D space of an analysed fundus image. The value of the normal retinal fractal dimension is around 1.7. Divergences from this number may reflect pathological states or a reduction in vessel density. Therefore, fractal analysis can help elucidate the significant biological changes in the early phases of disease (120,121).

Vessel width and pattern are not the sole indicators of disease. Developing automated identification of retinal abnormalities lie in diabetic retinopathy research, where this approach is crucial for timely intervention to prevent blindness. Two classes of abnormalities are distinguished: red lesions, including microaneurysms and retinal haemorrhages (122); and bright lesions like drusen, cotton-wool spots, and lipoprotein exudates (123). Different bright lesions are associated with different diseases; therefore, the call is to correctly distinguish between them, and therefore allow precise diagnosis and treatment.

1.9.2 Exploring the Interplay between Retinal Microcirculation and (Cerebro-) Cardiovascular Health

Fundus imaging serves as an instrument to examine the modifications in the retinal microcirculation. The retinal blood vessels exhibit comparable processes to those in the smaller vessels found in the brain and heart. This enables investigation of interconnection between alterations in the microvasculature of the retina and the risk factors or consequences of cardiovascular and cerebrovascular ailments, like in some of the large cohort studies such as Rotterdam Eye Study (RES), Multi-Ethnic Study of Atherosclerosis (MESA), Cardiovascular Health Study (CHS), Blue Mountains Eye Study (BMES), Beaver Dam Eye Study (BDES), and Atherosclerosis Risk in Communities Study (ARIC). The constriction of retinal arterioles and the dilation of venules have emerged as predictive factors for future cardiovascular events (124). Comparable relationships have also been observed in morbidity and mortality caused by cerebrovascular issues wherein retinal vessel alterations or retinopathies are linked to cerebral infarctions and an increased risk of clinical stroke (124,125).

Furthermore, modifications in retinal vascular caliber are intimately linked to established risk factors of the cardiovascular system, including obesity, hypertension, endothelial dysfunction, inflammation, atherosclerosis, and smoking. Elevated BP affects retinal microvasculature via the reduction of diameter retinal arterioles and widening diameters of retinal venules (126–129). Obesity is linked to constriction of retinal arterioles and/or dilation of retinal venules (126,130–132). Inflammation and related endothelial dysfunction dilate retinal venules and are associated with s systemic inflammatory diseases (for example metabolic syndrome) (126,130,131,133,134). Additionally, dilation of retinal venules was connected with atherosclerosis-related factors (such as reduction in HDL, elevated cholesterol and leukocytes, as well as higher waist-to-hip ratio, suggesting a potential

connection between venular widening and atherosclerosis (126,129,134). Lastly, smoking is consistently linked to wider retinal venules, indicating smoking-induced endothelial dysfunction (126,129,131,134).

Retinal arteriolar narrowing occurs when the blood vessels undergo functional (dysfunction of the endothelium) and structural (remodelling of the vasculature) changes, mainly studied in hypertension. Initially, the retinal arterioles can constrict to counteract BP elevation, resulting in blood flow reduction (myogenic vasoconstriction) (135,136). However, persistent high BP leads to arteriosclerosis: thickening and stiffening of the vessel walls, causing arteriolar narrowing and arteriovenous nicking followed by exudation: dilation of retinal vessels, resulting in severe retinopathy (135,136). As mentioned above, retinal venular dilation is associated with conditions involving inflammation and related endothelial dysfunction for instance smoking, obesity, or atherosclerosis. These conditions can activate leukocytes and disrupt the endothelial layer, increase intraluminal diameter, and lead to observable venular widening (126,130,134).

1.9.2.1 Can the Biological Effects of Light Therapy be pronounced in Retinal Microvasculature?

Light treatments have diverse biological influences from molecules and cells to whole tissues (137–142). Since the red light is able to penetrate deep into the tissue as well as due to its minimal haemoglobin and melanin mediated absorption, it is commonly employed for wound healing. (137). It stimulates DNA and RNA synthesis, enhances cell proliferation, and suppresses apoptosis in human fibroblasts (138). Light therapy also influences the activity of various proteins and has shown efficacy in treating conditions like diabetes, dengue fever, insomnia, hypertension, psychiatric illnesses, wound healing and seasonal affective disorder (SAD) (139–146).

One of the light treatment/therapy options is Maharishi light therapy (MLT), utilizing Vedic technology developed by Joachim Roller (147,148). Despite its reported enjoyable and relaxing effects, the research is limited (147–149). To date, no research investigating the interconnection between light therapy and retinal microcirculation parameters was noted. The pilot study of Saloň and colleagues (the article is part of the dissertation thesis) addressed this knowledge gap when they investigated for the first time the MLT effects on parameters of retinal microcirculation and hemodynamic (150). The results of their study suggest a

reflection of common microcirculation variations rather than the effect of light therapy itself (detailed information is presented below as the article is part of the dissertation thesis). Future studies with higher sample sizes longer light therapy employment and/or including patients suffering from different diseases are encouraged to reveal physiological insights of this therapy.

1.9.2.2 COVID-19 and Its Effect on Cardiovascular Health, Including Insights into Retinal Microvasculature

The Coronavirus disease 2019 (COVID-19) has been shown to have a profound impact on the cardiovascular system. Recent studies have indicated that COVID-19 can lead to endothelitis (151), which involves inflammation of the endothelial cells lining the blood vessels. Even more concerning is the finding that microvascular function may not fully recover even three months after the initial infection (152). This suggests that the virus may have long-lasting effects on the small blood vessels, impairing their ability to regulate blood flow effectively. Moreover, COVID-19 doesn't spare larger arteries either. Research has demonstrated that individuals who have contracted the virus exhibit lower vascular function and higher arterial stiffness (153,154). These changes in arterial health can have serious implications for overall cardiovascular well-being, potentially increasing the risk of conditions such as hypertension and atherosclerosis. In addition to these vascular effects, COVID-19 has been linked to a range of cardiovascular complications. These include but are not limited to acute coronary syndrome, myocarditis, arrhythmias, and valvular damage (155). These conditions can be life-threatening and have contributed to the morbidity and mortality elevation associated with COVID-19.

The pandemic has strained healthcare systems, leading to delayed cardiovascular care for those with pre-existing conditions, worsening the public health impact. There is an urgent need for research to understand the connection between COVID-19 and cardiovascular health, as well as for the development of quick and precise diagnostics to address these concerns, control the transmission of the virus, and reduce its impact on healthcare and society.

To date, there have been limited studies connecting COVID-19 with retinal microcirculation parameters. Saloň and colleagues conducted a pilot study, (the article is part of a dissertation thesis) addressing this knowledge gap. Their research delved into alterations in the cardiovascular system, encompassing both micro- as well as macro-vascular

parameters, in individuals following COVID-19. The study aimed to scrutinize these alterations at two-time points after discharge from hospitalization due to COVID-19. The first data collection slot took place either on the day of discharge from the hospital or on the tenth day following COVID-19-mediated hospitalization, and the second slot was sixty days after hospitalization. The alterations of HR, systolic BP, and microvasculature observed in our study suggest ongoing vascular adaptation may persist weeks or even months after overcoming COVID-19 (detailed information is provided below as the article is part of the dissertation thesis). Future research endeavors could include further evaluations throughout active infection as well as post-infection.

1.9.2.3 The Influence of Lower Body Negative Pressure on Cardiovascular Health and Retinal Microvasculature

Lower body negative pressure (LBNP) is a pivotal countermeasure for reversing the body fluids shift towards the head that is experienced by astronauts during prolonged microgravity exposure in spaceflight (156). In terrestrial settings, it is a valuable tool for evaluating the cardiovascular stability of individuals under central hypovolemia conditions, a phenomenon encountered during prolonged upright stand and haemorrhagic events. LBNP effectively shifts blood downwards, specifically to the pelvis, legs, and extravascular compartments, leading to a reduction in both central venous pressure as well as venous return (157). Interestingly, previous research has highlighted sex-related differences in orthostatic tolerance, with reports indicating that women may exhibit lower tolerance to central hypovolemia (158). While the concept of sex-based differences in central hypovolemia tolerance is well-established (159,160), the underlying mechanisms contributing to this disparity remain incompletely understood.

Although the impact of LBNP on various physiological reactions especially in large arteries has been extensively documented, a significant gap in the literature concerning its effects on microvasculature exists. Studying these effects in important, as prior research has indicated that alterations in the microcirculation, especially within the small retinal vessels, may precede changes observed in larger vessels (161–170). While previous investigations have linked retinal microcirculation with conditions such as sepsis (171–175), none have specifically addressed the implications of LBNP on microvascular dynamics. It is essential to recognize that, even though there are likenesses in the hemodynamic changes caused by septic

shock and hypovolemia, they are not identical.

Saloň and colleagues conducted a study (the article is part of a dissertation thesis) to explore the impact of varying levels of LBNP and gender differences on retinal microcirculation in a cohort of young, healthy individuals (176). This research aims to provide valuable insights into the intricate relationship between LBNP, microvascular responses, and sex-specific variations in cardiovascular adaptation. While LBNP employment did not show alterations in parameters of retinal microvasculature between sexes (detailed information is provided below as the article is part of the dissertation thesis), further investigation at higher LBNP levels, including those inducing presyncope is required, to elucidate dynamics of retinal vasculature during collapse of cardiovascular system as seen in hypovolemic shock or severe haemorrhage. Future research may also incorporate measurements of retinal blood flow, velocity, and vessel diameters to provide more complex insights, with an emphasis on collecting additional data to reveal the molecular mechanisms responsible for running of these adaptations.

1.10 Retinal Vascular Function in Health and Disease (Project Preview)

The dissertation objective is to investigate alterations in parameters of retinal microcirculation in various populations of healthy people as well as patients with different diseases (outlined in the Aims and Hypotheses section). The twelve studies/projects are partially included in this dissertation topic. These studies encompass six healthy populations and six populations of patients (the specific included studies are listed below).

In addition, correlation analyses are conducted between retinal microcirculation and various cardiovascular risk factors, as well as other cardiovascular measurements, depending on the specific study. The primary focus of this work is to evaluate small repulsive microcirculation in the retina as a reflection of cardiovascular health using retinal imaging techniques (detailed described in the methodological sections of included articles). Previous research has demonstrated the interconnection between retinal cardiovascular and cerebral circulation, highlighting the predictive power of retinal microcirculation assessment. Retinal imaging provides a non-invasive, affordable, painless, and rapid approach to assessing cardiovascular health. Moreover, this approach has the potential to serve as an additional clinically recognizable biomarker, enhancing the precision of cardiovascular health evaluation.

As this dissertation follows a cumulative thesis format, three first-author publications related to the dissertation topic are included within it. The specific studies/projects included in this dissertation are listed below, with the three presented in this thesis document highlighted in bold:

Six included studies with healthy populations:

1. Analysis of Retinal Blood Vessel Diameters in Pregnant Women Practicing Yoga
2. Ten days of bed rest modulates retinal microvascular circulation.
3. **Vascular responses following light therapy: a pilot study with healthy volunteers.**
4. HAPHC: Health & Academic Performance with Happy Children
5. **Vascular Stimulus Response in Health and Disease: A Longitudinal Study (VESSELS)**
6. Effect of Individualized Artificial Gravity Combined with Cycling on Cardiovascular, Cardio-postural, and Cerebral Responses in Healthy Males and Females

Six included studies with populations of patients:

1. The Effect of Transcendental Meditation and Yoga on Microcirculation in Cardiac Rehabilitation Patients – a pilot study
2. **A pilot study: Exploring the influence of COVID-19 on cardiovascular physiology and retinal microcirculation.**
3. A Pilot Study: Hypertension, Endothelial Dysfunction and Retinal Microvasculature in Rheumatic Autoimmune Diseases
4. A trial investigating ultra-long-acting basal insulins' flexibility around multiple spontaneous exercise sessions in people with type 1 diabetes: a head-to-head comparison of 2nd generation insulin Glargine U300 (IGlar-U300) to insulin Degludec U100 (IDeg-U100)
5. Does Nicotine reduce the risk of pre-eclampsia? A prospective study
6. COVID-19 and its Effects on Endothelium in HIV-Positive Patients in Sub-Saharan Africa: Cardiometabolic Risk, Thrombosis and Vascular Function (ENDOCOVID STUDY)

2 AIMS AND HYPOTHESES

CVDs impose an essential burden on modern society. They account for around one-third of worldwide deaths per year, and this number is sustained by an increasing trend. To fight the impact of CVDs, two key approaches are important: 1.) reducing cardiovascular risk factors, and 2.) improving diagnostics to enable fast and effective treatments. While risk factors such as smoking, eating habits, obesity, and physical inactivity play crucial roles in cardiovascular changes, natural physiological processes like pregnancy and aging also significantly affect cardiovascular health. Additionally, activities related to the effects of gravity on the body, leading to body fluid shifts, such as bed rest (commonly associated with older persons or hospitalization after injuries), and space missions (as space research grows in popularity), have an impact.

As a result, early and precise diagnosis of CVDs in at-risk individuals, along with the monitoring and treatment of patients already suffering from CVDs, have become more critical than ever before. Various methods can assess cardiovascular health, but many of these approaches are invasive, expensive, and time-consuming. Commonly used techniques involve evaluating blood markers, chest X-rays, echocardiography, and electrocardiograms. Other options include echocardiography, exercise or stress testing, cardiac catheterization, and CT/MRI scans.

Retinal imaging is an innovative, modern, and non-invasive physiological technique that involves analysing the small resistance vessels in the retina. As the blood vessels of the retina originate from the ophthalmic artery (the first branch of the internal carotid artery), and the retina itself shares development features with the neural system, as it develops from the diencephalon, this technique is often utilized as a surrogate method to assess cardiovascular or cerebrovascular health (177). Research has confirmed the role of small retinal vessels as predictors of changes in larger vessels (161–170). Constrictions of retinal arterioles and veins can predict a future increase in BP and subsequent hypertension development (163–165). Additionally, constriction of retinal arterioles and dilation of retinal venules are strong predictors of coronary heart disease and clinical stroke (166–170). Early detection of these signs could enable early diagnosis and treatment of a wide range of CVDs.

Based on the information above, the following aims and hypotheses have been made:

Aim #1: Examine how microvasculature in the retina changes in healthy persons in different populations.
Hypothesis #1: Retinal measurements will be different in healthy persons in different populations.

Aim #2: Assess how retinal microvascular changes over time in patients in different populations.
Hypothesis #2: Retinal measurements will be sensitive enough to capture changes in vascular health over time in patients in different populations.

Aim #3: Assess how microvascular changes in the retina correlate with changes in vascular function occurring in other arteries (e.g., brachial artery, carotid artery).
Hypothesis #3: The changes in retinal microvasculature will precede changes in big vessels.

Aim #4: Assess how retinal microvascular changes are correlated to known cardiovascular risk factors (e.g., age, BMI, ethnicity, physical activity).
Hypothesis #4: There will be correlations between retinal microcirculation parameters and cardiovascular risk factors.

3 CUMULATIVE DISSERTATION

This paragraph of the dissertation thesis includes one by one all three original, first-authored publications which are parts of this cumulative dissertation thesis. The consent to use the research work as a part of this cumulative thesis was obtained and confirmed by signatures from all co-authors within these three articles. Furthermore, the publisher and research journals publishing these scientific works provided consent to use these articles as a part of the present cumulative dissertation. Although, to keep the high quality and readability of these three articles below were placed as jpeg files, in case of necessity their original versions are easily traceable and available online.

Journal of
Clinical Medicine

MDPI

Article

Vascular Responses following Light Therapy: A Pilot Study with Healthy Volunteers

Adam Saloň [1,2], Bianca Steuber [1], Ruslan Neshev [1], Karin Schmid-Zalaudek [1], Patrick De Boever [3], Eva Bergmann [4], Rainer Picha [5], Per Morten Fredriksen [2], Benedicta Ngwechi Nkeh-Chungag [6] and Nandu Goswami [1,7,*]

[1] Gravitational Physiology and Medicine Research Unit, Division of Physiology, Otto Loewi Research Center, Medical University of Graz, 8036 Graz, Austria
[2] Faculty of Health and Social Sciences, Inland Norway University of Applied Science, 2624 Lillehammer, Norway
[3] Centre for Environmental Sciences, Hasselt University, 3500 Hasselt, Belgium
[4] Meditation Center in Silkeborg, Moerksoevej 71, 8600 Silkeborg, Denmark
[5] Rehabilitation Center for Cardiovascular Disease, 8061 St. Radegund, Austria
[6] Department of Biological and Environmental Sciences, Faculty of Health Sciences, Walter Sisulu University PBX1, Mthatha 5117, South Africa
[7] College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai P.O. Box 505055, United Arab Emirates
* Correspondence: nandu.goswami@medunigraz.at; Tel.: +43-316-385-73852; Fax: +43-316-385-79005

Abstract: (1) Background: Studies have reported the effectiveness of light therapy in various medical conditions. Our pilot study aimed to assess the effect of Maharishi light therapy (MLT) on physiological parameters, such as the heart rate (HR), HR variability (HRV), blood pressure (BP), BP variability (BPV), and the retinal microvasculature of healthy participants; (2) Methodology: Thirty (14 males and 16 females) healthy, non-smoking participants between 23 and 71 years old (46 ± 18 years) were included in this randomized crossover study. Each participant was tested with a placebo (using LED light) and gem lights, 24 h apart. Hemodynamic parameters were recorded during the session, and 24 h heart rate and BP levels were assessed via mobile devices. Retinal vascular responses were captured with fundus images and the subsequent analysis of retinal vessel widths. A linear model, using repeated measures ANOVA, was used to compare the responses across the sexes and to assess the effect of the MLT; (3) Results: Changes in the central retinal artery equivalent (CRAE) ($p < 0.001$) and central retinal vein equivalent (CRVE) ($p = 0.002$) parameters were observed. CRAE and CRVE decreased under MLT and increased under the placebo condition from before to after. However, the baseline values of the participants already differed significantly before the application of any therapy, and the variation in the retinal vessel diameters was already large in the baseline measurements. This suggests that the observed effect results may only reflect naturally occurring fluctuations in the microcirculation and not the effect of MLT. Furthermore, no significant effects were observed in any other investigated parameters; (4) Conclusion: Our study with healthy participants finds significant changes in retinal parameters, but the biological variation in the baseline measurements was large to begin with. This suggests that the observed effect results only reflect naturally occurring fluctuations in the microcirculation and not the effect of MLT. However, in the future, larger studies in which MLT is applied for longer periods and/or in patients with different diseases could discover the physiological impacts of this type of therapy.

Keywords: cardiovascular health; healthy volunteers; hemodynamics; retinal imaging; phototherapy; light therapy

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1. Introduction

The disturbance of cardiovascular physiological processes is the foundation of cardiovascular morbidity and mortality. There are many risk factors, including high blood

pressure, high blood cholesterol, smoking, diabetes, and others, that contribute to the development of cardiovascular disease (CVD). Aging reduces the elasticity of arteries, which decreases the flow of blood and oxygen, and can result in a permanently high blood pressure [1]. High cholesterol intake increases the level of LDL cholesterol and contributes to atherosclerosis development, resulting in narrowed arteries and hypertension [2]. Moreover, smoking increases cholesterol, LDL, and triglycerides, but decreases HDL [3]. The status of diabetes increases the level of oxygen radicals and reduces the level and bioavailability of NO, which decreases the elasticity and narrowing of blood vessels [4].

Yoga and meditation practices are considered to be non-invasive approaches to improving cardiovascular health [5,6]. These types of Vedic techniques also include light therapy. However, evidence and claims about the effect of light therapy are limited and insufficient [7–9].

It is suggested that light treatments produce biological effects in molecules, living cells and tissues [10–15].

One of the most common health issues treated by phototherapy is wound healing. Red light is typically used for wound healing due to its increased penetration through tissue and its lower absorption by hemoglobin and melanin [10]. The irradiation of human fibroblasts with red light (λ = 628 nm) leads to an increase in DNA and RNA synthesis with gene expression upregulation, with most of them playing a role in increasing cell proliferation and suppressing apoptosis [11].

Other proteins whose activity can be affected by phototherapy include superoxide dismutase (max in 644 nm), glucose oxidase (464 nm), cholesterol esterase and cholesterol oxidase, and lipase (400 nm) [12,13].

The effectiveness of therapy that uses wavelengths in the visible region has been shown for various medical conditions, such as dengue fever, insomnia, diabetes, psychiatric illnesses, hypertension, seasonal affective disorder (SAD), immunity, hyperacidity, cutaneous wound healing, chronic joint diseases and inflammation [12,14–19].

Thrombocytopenia is a typical symptom in patients with dengue fever. Chromotherapy by red-colored radiation (644 nm) has been shown to inhibit platelet degradation [15]. Furthermore, the limited options for treating insomnia during pregnancy has led to the application of chromotherapy. It has been shown that the color turquoise (495 nm) improves sleep, and decreases fatigue and drowsiness during pregnancy [16]. In turn, a light therapy with green, yellow or orange light can be helpful in controlling diabetes [19]. Bright Light Therapy has been proven to be an adequate countermeasure for SAD and other mental health disorders [18]. A detailed discussion of the different methods is summarized in a paper by Azeemi and coworkers [17]. Despite the presentation of different studies focused on light therapy, most of them struggle with problems regarding the sample size and heterogeneity, so further research is needed [8].

Maharishi light therapy uses Vedic technology, which was first applied more than 30 years ago by Joachim Roller, an apprentice of Maharishi Mahesh Yogi [20,21]. Following Maharishi's guidance, a jewelry designer, Joachim Roller, developed gem beamers. Since 2007, the gem beamer technology has been used around the world. Maharishi light therapy (MLT) focuses on light passing through gems (such as diamonds, emeralds, and rubies) and it is applied to specific areas of the body. People report that the therapy is enjoyable, deeply relaxing, and refreshing for their mind and body [20,21]. To date, however, only a limited number of articles have evaluated the impact of light therapy on physiology, and none have focused on Maharishi light therapy. The lack of knowledge about Maharishi light therapy is evident and, as Travis et al. noted, "an assessment of the impact of light therapy on the physiology of the human body is necessary" [22]. This pilot study investigated the effects of MLT on physiological parameters within a triple-blinded randomized, crossover study. We assessed hemodynamic parameters, heart rate variability (HRV), blood pressure variability (BPV), and microvascular responses in healthy participants.

2. Materials and Methods

The investigations of this study were performed at the Medical University of Graz, Austria. The study was submitted to and approved by the Ethics Committee of the Medical University of Graz, Austria (EK: 30-515 ex 17/18). Data collection was performed in accordance with good clinical practices and following the WMA Declaration of Helsinki (2013). Every participant received detailed information about the study protocol and provided written consent.

2.1. Participants

In total, 30 (14 males and 16 females) healthy, non-smoking participants were enrolled in this study. They were between 23 and 71 years of age (46 ± 18 years), of 160–185 cm in height (170.9 ± 7.8 cm), and 51–130 kg in weight (71.0 ± 18.7 kg) (Table 1). The exclusion criteria were individuals who smoked, consumed alcohol on a regular basis, had psychological problems, had heart disease, were on medications that influence cardiac parameters (e.g., beta blockers), or were pregnant.

Table 1. Characteristics of the study participants (n = 30). Data give mean ± SD (range).

Characteristics	Males = 14		Females = 16	
Age (years)	(46 ± 20)	23–71	(46 ± 17)	24–71
Height (cm)	(176.7 ± 6.2)	166–185	(165.8 ± 5.0)	160–179
Weight (kg)	(82.5 ± 19.5)	56–130	(61.1 ± 10.9)	51–85
BMI (kg/m^2)	(26.5 ± 6.8)	20–45	(22.2 ± 3.6)	19–32

2.2. Study Design

This was a triple-blind, randomized, crossover study. Neither the administrator of the light therapy nor the participant receiving the treatment knew about the order of the MLT vs placebo intervention. The measurements took two days (48 h, 24 h per study condition). Participants were randomly assigned to two appointments within two successive days. The participants who received MLT on the first day crossed over to the second intervention (placebo) on the second day and vice versa. All measurements were obtained within two days (48 h). The placebo intervention was nonrecognizable from MLT with real gems. The study was conducted to investigate the effect of MLT on the physiological parameters and microvasculature. Data analysis was performed offline by a person (R.N.) who had no knowledge of the treatments/condition (MLT or placebo) on a given day. Randomization was performed by using a free, demo version of online software https://www.randomizer.at (accessed on 1 April 2019).

2.3. Light Therapy Application Device

Gems that emitted different colors of light were used. A device comparable to the one that administered the MLT was also made, but only LED lights of similar colors to the ones used in the MLT were used. It was not possible to differentiate the gems- vs the placebo light-administering device.

2.3.1. Light Therapy Pens

The MLT pens were around 1.5 cm in diameter and 15 cm long, powered by a battery. The light passed through 13 different gems: amber, amethyst, blue sapphire, carnelian, cat's eye, coral, diamond, emerald, green tourmaline, pearl, ruby, yellow sapphire, and zircon. The light was projected through the gems to the core of the body (abdomen, chest). The incident lights were focused in circles, whose size indicated how far the pens were placed from the participant's body.

2.3.2. Placebo Light Pens

The placebo light pens were of the same shape, color, and material as the authentic light therapy pens. They projected light through colored glass instead of gems. The light was similar in color and diameter to that in the light therapy pens, and the real application device and placebo version could not be distinguished from each other.

2.4. Light Therapy Protocol

As mentioned in the study design, the participants were given two appointments, one for the MLT with real gems and one for the placebo intervention. Each intervention was 24 h apart and each participant was investigated under both conditions, using MLT and placebo light therapy, in a randomized crossover design. The MLT light therapy was performed by an experienced practitioner (E.B.), who had no knowledge of who received which intervention (MLT or placebo) on a given day.

After the participant arrived, they received detailed information about the study protocol and provided written consent. The protocol began with the retinal imaging (5 min). Thereafter, electrodes were placed on the participant's body, and with lying them in a supine position, the 10 min baseline values of the hemodynamic parameters (TFM, Task Force Monitor) were recorded. The protocol continued with an interview with the practitioner of the study (20 min) (information not included in manuscript). Following, the participants were exposed to either MLT or placebo light intervention, using the same apparatus; however, instead of gem beams, LED beams with the same light colors were used (20 min). The administration of the two conditions was randomized and blinded, and both conditions (–MLT/placebo light) were administered on successive days. Physiological measurements using TFM (epochs T1–T25) were collected during the whole intervention, as well as during recovery in the supine position (5 min). After recovery, BP and HRV devices for continual 24 h measurements were placed on the participant (10 min). The devices measured the BP and HR of the participants over the following 24 h until the participant underwent the alternating intervention. These measurements enabled the evaluation of a potentially longer light therapy impact. Before the participant left, the second retinal imaging procedure was performed (5 min). Participants returned the next day (after 24 h) to receive the other condition/intervention. The procedure for the light therapy protocol is described in Figure 1.

2.5. Physiological Measurements

All measurements were collected using a Task Force Monitor® (TFM, CNSystems, Graz, Austria). BP (upper arm oscillometry and finger plethysmography), HR (3-lead ECG) and thoracic impedance were measured for the purposes of hemodynamic monitoring. For detail of the electrode's placement, see Trozic et al., (2020) [23].

The collected physiological data were analyzed by calculating the means of 30 s epochs. The last 30 s of the baseline were taken as a reference (epoch T0), and then analogous with the light intervention, which lasted between 13 and 30 min. In order to remain consistent, the first 25 epochs of the MLT/placebo were taken: T1-T25. Only means with more than 85% valid values (more than 25 s) were taken, and the rest were set to missing values in order to reduce the bias that could have arisen in any of the analyses [24]. The means and the plots of the epochs were generated using MATLAB R2018a (Version 9.4.0, The MathWorks Inc., Portola Valley, CA, USA). The totals of the 26 epochs were then analyzed using SPSS (Version 26.0, SPSS Inc., Armonk, NY, USA).

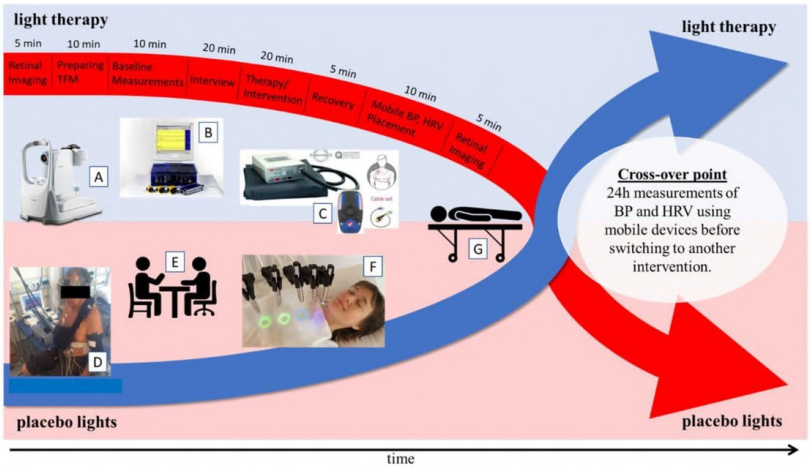

Figure 1. Simplified light therapy study protocol. The red and blue arrows represent the crossover of the two interventions (MLT or placebo light) of the study group. The red arrow is used to display the flow of the protocol procedures and the respective time slots. The individual small images (**A**–**G**) in the core of the picture display the equipment and procedures used during the experiment (explained below). The crossover point describes the timing between the switch of therapies. (**A**)—retinal camera used to capture retinal images, (**B**)—task force monitor used to measure all physiological parameters, (**C**)—mobile devices used to measure 24 h variability in blood pressure (**left**) and heart rate (**right**) (detailed in Section 2.6), (**D**)—baseline measurements of physiological parameters using TFM, (**E**)—interview during the protocol, (**F**)—therapy/intervention (MLT or placebo light), and (**G**)—recovery in the supine position.

2.6. Heart Rate Variability and Blood Pressure Variability Measurements

A portable ECG-measurement device (eMotion Faros, Biosign, Ottenhofen, Germany) was used to record the participants' ECG over the course of 24 h after each intervention (MLT or placebo). These data were then applied to a long-term (24 h) HRV analysis. A similar dataset with the participants' BP measurements (TM-2430, A&D) over the 24 h after each intervention was also acquired and analyzed.

The HRV data were analyzed by removing any large motion artifacts with the help of the Symlets 4 (sym4) wavelet transformation. Then, the Pan-Tompkins algorithm was used to detect the R-peaks in the 24 h ECG signal and build the normal-to-normal intervals (NN intervals) of the participants [25]. The NN intervals were analyzed with statistical time domain methods—e.g., SDNN (standard deviation of all NN intervals), SDSD (standard deviation of differences between adjacent NN intervals), etc. [25]. The parameters that were analyzed are all in the time domain as the frequency domain parameters and are more prone to errors due to artifacts. Furthermore, the time domain parameters correlate directly to frequency domain parameters and therefore can be used as a measure for those [25]. The 24 h BPV data were partly analyzed externally. The University of Minnesota provided the sphygmochron data, which contain the MESOR; this is an adjusted 24 h mean, which was obtained by fitting a cosine model to the original data [26].

2.7. Retinal Measurements

The retinal images (resolution of 1536 × 1536) of the right eye were obtained from each of the participants before and after each intervention, as indicated in the protocol. To capture the optic disc-focused retinal images, a non-mydriatic digital retinal camera, the Canon CR-2 (Canon Medical Systems Europe B.V., Zoetermeer, The Netherlands), was used. Retinal images were arranged, organized, and prepared for analysis. A trained operator,

without any previous knowledge about the details of the study, used the semi-automated MONA REVA software (VITO, Mol, Belgium; [27]) to analyze the retinal images. The software automatically processed the retinal images and analyzed the diameters of retinal microvessels in areas 0.5 to 1 of the optic disc radius from the optic disc margin. Post-processing, including double thresholding, blob extraction, the removal of small connected regions, and filling holes, was performed. Subsequently, the vessels (arterioles, venules) were checked, corrected, and labeled by the grader. The Parr–Hubbard–Knudtson formula, which uses the 6 largest retinal arterioles and the 6 largest retinal venules, was used to calculate three retinal parameters: central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE), and artery-to-vein ratio (AVR) [28].

2.8. Statistical Analysis

The physiological parameters obtained during the study were first analyzed by the Shapiro–Wilk normality test to check the distribution of the data. Afterward, repeated measures one-way and two-way ANOVA, and analyses of the effects of the different covariates on the linear model, were used. The repeated measures ANOVA was performed for all the participants pooled together and once additionally for sex as a between-subject factor. Three covariates were additionally considered for the analysis: age, weight, and height. Any between-subject factors and covariates that were found not to be statistically significant were removed from the further analysis. All statistical tests were performed with the proper assumptions checks (e.g., Shapiro–Wilk test for normality), and any inconsistencies were removed. The data are presented as means ± standard deviation.

The data from the 24 h HRV and BPV measurements were checked for normal distribution with the Shapiro–Wilk test. The HRV parameters were non-normally distributed and were, therefore, analyzed using the non-parametric Wilcoxon test. The data are presented as means ± standard deviation. Because the BP variability parameters were normally distributed, the standard arithmetic means to perform *t*-tests were used. The data are presented as means ± standard deviation.

The normal distribution of retinal parameters was also analyzed using the Shapiro–Wilk test, and a repeated measures ANOVA was applied to analyze the data; this included *before* and *after,* and the MLT *or* placebo as the repeated measures factor, testing all participants, triple-blinded, under both conditions in a randomized design. To evaluate specific effects, respective post hoc tests were performed, correcting the alpha level according to Bonferroni.

3. Results

Thirty participants were recruited for this crossover study. Five participants were excluded from the retinal imaging analysis due to the very low quality of the retinal images obtained from them. Table 1 summarizes the basic characteristics of the study participants.

3.1. Physiological Parameters Obtained with the Task Force Monitor

None of covariates age, weight, and height, showed an effect on the linear model. Thus, the parameters were excluded from the analysis. The cardiovascular parameters were assessed at six different time points (T0–T5, see Supplementary Table S1).

Of all the hemodynamic parameters examined, only one significant effect was found for HR ($p = 0.044$), indicating a lower HR (mean ± SD) under the placebo condition than under the MLT condition (mean ± SD); however, the difference in the means was less than 2 bpm (1.616) (Supplementary Table S1).

3.2. Heart Rate and Blood Pressure Variability Measurements

No statistically significant results were found in the 24 h HRV and BPV measurements (Supplementary Tables S2 and S3).

3.3. Retinal Measurements

In this study, retinal data were available for 25 participants, comparing images taken *before* and *after* the MLT and placebo interventions. Significant differences were found in the change in the CRAE ($p < 0.001$) and CRVE ($p = 0.002$) before and after the MLT/placebo intervention (Table 2 and Supplementary Table S4).

Table 2. The results of retinal microcirculation measurement. The means, and standard deviations of the retinal parameters of participants ($n = 25$). CRAE (central retinal artery equivalent), CRVE (central retinal vein equivalent), and AVR (artery-to-vein ratio). Data are shown as means (SD).

Retinal Parameter	Intervention	Before	After	F-Value, *p*-Value
CRAE (µm)	MLT	148.9 (18.1)	145.6 (16.3)	F(1,24) = 17.004, $p < 0.001$
	Placebo	145.5 (18.1)	147.5 (17.5)	
CRVE (µm)	MLT	222.7 (20.6)	218.3 (19.8)	F(1,24) = 11.999, $p = 0.002$
	Placebo	219.5 (22.1)	221.9 (20.2)	
AVR	MLT	0.668 (0.047)	0.668 (0.051)	F(1,24) = 0.069, $p = 0.796$
	Placebo	0.663 (0.049)	0.665 (0.053)	

While under MLT the CRAE and CRVE decreased from before to after, the CRAE and CRVE increased under the placebo condition. However, the measures of the participants already differed significantly before the start of any of the interventions. As can be seen in Table 2, the variation in the retinal vessel diameters was large already in the baseline measurements. Hence, the significant effect results from the opposing change in CRAE (and CRVE) under MLT compared to the placebo, which might only reflect naturally occurring fluctuations in the microcirculation, rather than an effect of MLT.

4. Discussion

In this study, we investigated the effect of MLT on cardiovascular physiology. We observed that CRAE and CRVE became smaller after MLT, and to the same extent, wider after placebo therapy. However, CRAE after MLT reached values similar to those achieved under the baseline condition before the placebo light therapy. Furthermore, baseline values of the same individual differed to the same extent before the application of any therapy, suggesting that the observed effect is only due to naturally occurring fluctuations in the opposing direction. Furthermore, no significant effects on HRV and BPV were found. To our knowledge, no previous studies have examined the effect of light therapy on retinal microcirculation.

Light therapy, as well as yoga or meditation, is a type of energy therapy in which the belief that there are energy fields that flow through and around your body plays an important role. One recent study investigated the effect of a 4-week cardiac rehabilitation intervention on 2 groups of patients, using typical exercise therapy (control group) and typical exercise therapy plus transcendental meditation (intervention group) to determine both cardiovascular and muscular responses [29]. They observed a significant reduction in systolic blood pressure and a nearly significant reduction in HR; they also observed a significant elevation in the RR interval after 4 weeks of rehabilitation, without interactions between the groups [29]. This study also examined the retinal microcirculation parameters. It included one more follow-up measurement and another rehabilitation group of patients (typical exercise therapy and yoga exercise). However, no significant results were observed during the study, as well as between the different study rehabilitation groups (data not published).

Several previous studies showed a decrease in the HRV, and a switch in the sympathovagal balance to the sympathetic side after light therapy [30–32]. We did not observe changes in the HRV parameters.

The narrowing of vessels limits blood flow, hence increasing BP within the walls of the blood vessels [33]. However, we did not find changes in BP in our study. Light therapy in the visible range spectrum can affect photosensitive molecules, such as ATP, superoxide

dismutase, or cytochrome C oxidase, which then affect the redox balance in the cells [11–13]. Current studies suggest that light therapy leads to an increase in mitochondrial activity, ROS production, as well as NO and, therefore, in vasodilation [11,34–36]. We did not find that light therapy had any significant effects on systolic blood pressure (SBP) or diastolic blood pressure (DPB). A pilot study that included 44 hypertensive subjects investigated the effect of laser acupuncture on BP, body weight, and HRV [37]. The low-level laser treatment (90 days, at least 12 treatments per subject) caused a significant reduction in both SBP and DBP [37]. Therefore, in our case, a short period of light therapy and the fact that our study group included only healthy individuals could be reasons for the lack of significant results found. To support the light therapy findings above, Heiss and colleagues, in a randomized crossover study that included 14 healthy male subjects, investigated the 2-day effect of monochromatic blue light or blue light with a filter foil (control light) on cardiovascular health. One light session took 30 min [36]. They saw that monochromatic blue light causes a decrease in SBP and arterial stiffness, and improves endothelial function [36]. Moreover, they also observed increased blood flows and increased levels of nitric oxide species as clear signs of vasodilatation, which is in contrast with the present study. However, our baseline values in the same individual differed to the same extent before the application of any therapy, suggesting that the observed effect is only due to naturally occurring fluctuations. Furthermore, this was not confirmed by any significant effects in other investigated parameters.

Regarding HR, a study that included 7 participants observed a decrease in the HR after 10 min of blue light (456 nm) exposure [30]. Another study investigating free-living trends in sleep and recovery found that the mean HR was significantly lower the night after light therapy [38]. The study included 12 athletes, who were exposed to visible red (660 nm) and near-infrared (NIR, 850 nm) light in an average of 8.5 $\pm$ 7.5 sessions/athlete, while one session took 20 min. No more than 2 sessions and no sessions on consecutive days were allowed. Distinct from their study, we observed a higher HR after MLT ($p = 0.044$). However, the significant effect was so small that the difference in the means was less than 2 bpm (1.616), so it could be easily caused by any small variation during the flow of the protocol. Unfortunately, the statistical tests only detect relative differences and no absolute values, such as 2 or 20 bpm. Therefore, these results must be interpreted very carefully and should be repeated in future research.

No significant effects on HRV and BPV were found in our study. The crossover study of Travis et al. found that MLT had a significant effect on the subjective feeling of wellness in 18 individuals with experience in meditation [22]. The design of their study was similar to ours, but all subjects were long-term meditation practitioners, and the favorable results of the study could be easily caused by the effect of meditation rather than by MLT itself.

The same study that observed a decrease in HR after 10 min of exposure to blue light (456 nm) also noted a decrease in HRV [30]. Yuda et al. found a significant decrease in high frequency (HF) and an increase in the low frequency (LF)/HF ratio as an indicator of reduced parasympathetic activity; this was due to the effect of 6 min of blue, red, and green light exposure [31]. Another study included 20 participants and found that 10 min of exposure to red light increases LF/HF ratio and LF, hence increasing sympathetic activity [32]. On the other hand, blue light causes a reduction in LF and the LF/HF ratio, and an increase in HF causes more cardiac relaxation via parasympathetic activity [32].

While we could not find any additional studies that examined the effects of light therapy on HRV and BPV, the available literature from yoga studies show that yoga exercise leads to a significant increase in HRV parameters, especially those associated with the vagal tone [39–41]. Khattab and colleagues observed an increase in HRV as the effect of a 5-week (once week/90 min) yoga program in 11 healthy (7 women and 4 men, mean age: 43 $\pm$ 11; range: 26–58 years) study participants [39]. Similarly, Papp and colleagues noticed increased vagal tone and reduced sympathetic activity after an 8-week yoga program [40]. In agreement with the previous two studies, a shift in the sympathovagal balance in favor of parasympathetic dominance was observed after meditation practice (once per day, 4 times

per week for one year) [41]. However, most studies investigating the effect of yoga on HRV are completed in India, struggle with sample size, are poor in design and quality, and use a range of heterogeneous measures [41–44]. Overall, the effect of yoga on HRV parameters appears to be beneficial, and shifts the autonomic regulation on the parasympathetic side; however, more research, especially from western countries, is needed in order to confirm these findings.

5. Limitations

There are some limitations to this study: (1) We do not have detailed information about the gem lights used for the MLT or knowledge about wavelengths. It is difficult to compare our results with previous research and an overall interpretation of the results must be performed carefully; (2) According to previous studies about the effects of light therapy on human health, the majority of the studies applied light for longer periods of time than our study (the length of the intervention in our protocol was set according to the recommendations of the MLT practitioner), and it is possible that we did not see changes in any other parameters because the application time of the light was short; (3) While our study investigated cardiovascular physiology parameters in healthy participants, most studies used light therapy as a treatment (e.g., depression, hypertension, wounds healing, etc.), when the effect of light therapy could be possibly more pronounced by the imbalance of the body; (4) As it is presented in the results section, the significant effects might only reflect naturally occurring fluctuations in the microcirculation, rather than an effect of MLT, and, therefore, these results need to be interpreted very carefully; and (5) The observed caliber of the retinal microvasculature could also be influenced by errors introduced while measurements were being taken. We do not believe that this is the case, as all the retinal measurements were carried out by the same person (A.S.).

6. Conclusion and Future Directions

The present pilot study observed changes in CRAE ($p < 0.001$) and CRVE ($p = 0.002$). CRAE and CRVE decreased under MLT and increased under the placebo condition from before to after. Hence, the significant effect results from the opposing change in CRAE (and CRVE) under MLT, compared to the placebo. However, because the baseline values of the participants already differed significantly before the application of any therapy, and the variation in retinal vessel diameters was large already in the baseline measurements, the observed effect results may only reflect naturally occurring fluctuations in the microcirculation and not the effect of MLT. Thereby, the results observed in the present pilot study should be treated with caution. No other significant changes in the measured physiological parameters were found to support the findings related to retinal microcirculation. It is possible that the application of the MLT was of a rather short duration.

The present pilot study investigated the effects of MLT on healthy volunteers. In the future, larger studies, in which MLT is applied for longer periods of time and/or in patients with different diseases (e.g., depression, hypertension), could discover the physiological impacts of this type of therapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12062229/s1, Table S1: Overview of the cardiovascular parameters. Displayed as mean (±SD) of the first 6 epochs—the last 30 s of baseline (T0) and the first 2.5 min of the light therapy (T1–T5), of all participants, pooled together ($n = 30$). HR (heart rate), SBP (systolic blood pressure), DBP (diastolic blood pressure), MAP (mean arterial pressure), SV (stroke volume), CO (cardiac output), TPR (total peripheral resistance); Table S2. The 24 h HRV parameters of two interventions (MLT or placebo light) of the study group. Displayed as means (±SD), z-score and p-value. NN50 count (number of pairs of adjacent NN intervals differing by more than 50 ms in the entire recording), pNN50 (NN50 count divided by the total number of all NN intervals), SDNN (standard deviation of all NN intervals), RMSSD (the square root of the mean of the sum of the squares of differences between adjacent NN intervals), SDSD (standard deviation of differences between adjacent NN intervals), SDANN (standard deviation of

the averages of NN intervals in all 5 min segments of the entire recording), SDNN index: (mean of the standard deviations of all NN intervals for all 5 min segments of the entire recording); Table S3. An overview of the 24 h blood pressure parameters (mean ± SD) of all participants, as well as the results of the paired *t*-test. It shows the parameters as means ± standard deviation and the results from the paired *t*-tests, presented as the T-score, the degrees of freedom, and the p-value. HR (heart rate), SPB (systolic blood pressure), DPB (diastolic blood pressure), MESOR (midline estimating statistic of rhythm); Table S4. Within-subject effects for the total cohort of participants without between-subject effects or covariates. CRAE (central retinal artery equivalent), CRVE (central retinal vein equivalent), AVR (artery-to-vein ratio).

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data are contained within the article.

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Supplementary Materials

Table S1. Overview of the cardiovascular parameters. Displayed as mean (± SD) of the first 6 epochs—the last 30 s of baseline (T0) and the first 2.5 min of the light therapy (T1–T5), of all participants, pooled together ($n = 30$). HR (heart rate), SBP (systolic blood pressure), DBP (diastolic blood pressure), MAP (mean arterial pressure), SV (stroke volume), CO (cardiac output), TPR (total peripheral resistance).

Cardiovascular Parameters	Intervention	First 6 Epochs—The Last 30 s (Mean ± SD)						*p*-Value
		T0	T1	T2	T3	T4	T5	
HR	MLT	68 (11)	63 (9)	64 (9)	63 (9)	63 (9)	64 (10)	0.044
(bmp)	Placebo	66 (10)	63 (11)	63 (12)	62 (10)	63 (10)	63 (11)	
SBP	MLT	118 (15)	113 (21)	114 (20)	116 (19)	116 (19)	116 (19)	0.32
(mmHG)	Placebo	120 (19)	116 (23)	112 (17)	112 (16)	112 (16)	113 (16)	
DBP	MLT	73 (14)	68 (17)	69 (18)	70 (17)	70 (17)	69 (17)	0.671
(mmHG)	Placebo	75 (14)	71 (15)	69 (15)	69 (14)	69 (14)	70 (14)	
MAP	MLT	91 (14)	86 (18)	87 (18)	89 (16)	89 (16)	88 (16)	0.743
(mmHG)	Placebo	92 (15)	89 (18)	86 (15)	86 (15)	87 (14)	87 (14)	
SV	MLT	89 (26)	92 (28)	90 (28)	90 (28)	90 (27)	90 (26)	0.329
(ml)	Placebo	90 (28)	92 (29)	92 (31)	93 (32)	91 (30)	92 (30)	
CO	MLT	6 (2)	6 (2)	6 (2)	6 (2)	6 (2)	6 (2)	0.88
(l/min)	Placebo	6 (2)	6 (2)	6 (2)	6 (2)	6 (2)	6 (2)	
TPR	MLT	1293 (454)	1304 (569)	1318 (585)	1378 (610)	1353 (607)	1328 (526)	0.388
(dyne x s/cm^5)	Placebo	1347 (532)	1384 (647)	1334 (574)	1333 (576)	1357 (605)	1342 (594)	

1

Table S2. The 24 h HRV parameters of two interventions (MLT or placebo light) of the study group. Displayed as means (± SD), z-score and *p*-value. NN50 count (number of pairs of adjacent NN intervals differing by more than 50 ms in the entire recording), pNN50 (NN50 count divided by the total number of all NN intervals), SDNN (standard deviation of all NN intervals), RMSSD (the square root of the mean of the sum of the squares of differences between adjacent NN intervals), SDSD (standard deviation of differences between adjacent NN intervals), SDANN (standard deviation of the averages of NN intervals in all 5 min segments of the entire recording), SDNN index: (mean of the standard deviations of all NN intervals for all 5 min segments of the entire recording).

Parameters	Intervention	Mean (SD)	Z	*p*-Value
NN50 count (total number)	MLT	8208 (6718)	−0.751	0.452
	Placebo	7438 (7311)		
pNN50 (%)	MLT	14.87 (12.60)	−0.956	0.339
	Placebo	12.56 (11.33)		
SDNN (ms)	MLT	220.37 (71.66)	−0.934	0.350
	Placebo	206.5 (68.60)		
RMSSD (ms)	MLT	186.5 (122.14)	−0.114	0.909
	Placebo	166.4 (104.94)		
SDSD (ms)	MLT	186.5 (122.14)	−0.114	0.909
	Placebo	166.4 (104.94)		
SDANN (ms)	MLT	139.91 (50.26)	−0.296	0.767
	Placebo	140.73 (50.13)		
SDNN index (ms)	MLT	143.38 (83.10)	−0.296	0.767
	Placebo	127.14 (64.64)		

Table S3. An overview of the 24 h blood pressure parameters (mean ± SD) of all participants, as well as the results of the paired t-test. It shows the parameters as means ± standard deviation and the results from the paired t-tests, presented as the T-score, the degrees of freedom, and the *p*-value. HR (heart rate), SPB (systolic blood pressure), DPB (diastolic blood pressure), MESOR (midline estimating statistic of rhythm).

24 h Blood Pressure Parameters	Intervention	Mean (SD)	T	df	*p*-Value
MESOR SBP (mmHg)	MLT	131 (14)	0.797	26	0.433
	Placebo	130 (11)			
Mean SBP (mmHg)	MLT	133 (15)	0.911	26	0.371
	Placebo	132 (10)			
MESOR DBP (mmHg)	MLT	77 (7)	−0.09	26	0.931
	Placebo	78 (6)			
Mean DBP (mmHg)	MLT	79 (7)	0.351	26	0.728
	Placebo	79 (6)			
MESOR HR (bpm)	MLT	70 (7)	−0.49	26	0.628
	Placebo	71 (8)			
Mean HR (bpm)	MLT	72 (7)	0.101	26	0.921
	Placebo	72 (8)			

Table S4. Within-subject effects for the total cohort of participants without between-subject effects or covariates. CRAE (central retinal artery equivalent), CRVE (central retinal vein equivalent), AVR (artery-to-vein ratio).

Parameter	Effect		
	MLT-Placebo	Before-After	MLT-Placebo x Before-After
CRAE	$F(1,24) = 0.710$, $p = 0.408$, $\varepsilon = 1$, partial $\eta^2 = 0.029$	$F(1,24) = 0.550$, $p = 0.465$, $\varepsilon = 1$, p. $\eta^2 = 0.022$	$F(1,24) = 17.004$, $p = 0.000$, $\varepsilon = 1$, p. $\eta^2 = 0.415$
CRVE	$F(1,24) = 0.020$, $p = 0.889$, $\varepsilon = 1$, partial $\eta^2 = 0.001$	$F(1,24) = 0.913$, $p = 0.349$, $\varepsilon = 1$, p. $\eta^2 = 0.037$	$F(1,24) = 11.999$, $p = 0.002$, $\varepsilon = 1$, p. $\eta^2 = 0.333$
AVR	$F(1,24) = 0.947$, $p = 0.340$, $\varepsilon = 1$, partial $\eta^2 = 0.038$	$F(1,24) = 0.017$, $p = 0.896$, $\varepsilon = 1$, p. $\eta^2 = 0.001$	$F(1,24) = 0.069$, $p = 0.796$, $\varepsilon = 1$, p. $\eta^2 = 0.003$

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A pilot study: Exploring the influence of COVID-19 on cardiovascular physiology and retinal microcirculation

Adam Saloň [a,h], Ruslan Neshev [a], Kaja Teraž [b,c], Boštjan Šimunič [b], Manca Peskar [b,d], Uroš Marušič [b,e], Saša Pišot [b], Luka Šlosar [b,e], Mladen Gasparini [f], Rado Pišot [b], Patrick De Boever [g], Karin Schmid-Zalaudek [a], Bianca Steuber [a], Per Morten Fredriksen [h], Benedicta Ngwenchi Nkeh-Chungag [i], Harald Sourij [j], Omar Šerý [k,l], Nandu Goswami [a,m,*]

[a] Division of Physiology & Pathophysiology, Otto Loewi Research Center for Vascular Biology, Immunology, and Inflammation, Medical University of Graz, Austria
[b] Science and Research Centre Koper, Institute for Kinesiology Research, Koper, Slovenia
[c] Faculty of Sport, University of Ljubljana, Slovenia
[d] Biological Psychology and Neuroergonomics, Department of Psychology and Ergonomics, Faculty V: Mechanical Engineering and Transport Systems, Technische Universität Berlin, Berlin, Germany
[e] Department of Health Sciences, Alma Mater Europaea – ECM, Maribor, Slovenia
[f] Department of General Surgery, General Hospital Izola, Izola, Slovenia
[g] Centre for Environmental Sciences, Hasselt University, Belgium
[h] Faculty of Health and Social Sciences, Inland Norway University of Applied Sciences, Lillehammer, Norway
[i] Department of Biological and Environmental Sciences, Faculty of Health Sciences, Walter Sisulu University PBX1, 5117 Mthatha, South Africa
[j] Internal Medicine, Division of Endocrinology and Diabetology, Interdisciplinary Metabolic Medicine Trials Unit, Medical University of Graz, Graz, Austria
[k] Laboratory of Neurobiology and Molecular Psychiatry, Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic
[l] Laboratory of Neurobiology and Pathological Physiology, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Veveří 97, 602 00 Brno, Czech Republic
[m] College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates

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ABSTRACT

Background: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) affects the cardiovascular system. The current study investigated changes in heart rate (HR), blood pressure (BP), pulse wave velocity (PWV), and microcirculation in patients recovering from Coronavirus disease 2019 (COVID-19) infection.
Methodology: Out of 43 initially contacted COVID-19 patients, 35 (30 males, 5 females; age: 60 ± 10 years; and body mass index (BMI): 31.8 ± 4.9) participated in this study. Participants were seen on two occasions after hospital discharge; the baseline measurements were collected, either on the day of hospital discharge if a negative PCR test was obtained, or on the 10th day after hospitalization if the PCR test was positive. The second measurements were done 60 days after hospitalization. The vascular measurements were performed using the VICORDER® device and a retinal blood vessel image analysis.
Results: A significant increase in systolic BP (SBP) (from 142 mmHg, SD: 15, to 150 mmHg, SD: 19, $p = 0.041$), reduction in HR (from 76 bpm, SD: 15, to 69 bpm, SD: 11, $p = 0.001$), and narrower central retinal vein equivalent (CRVE) (from 240.94 μm, SD: 16.05, to 198.05 μm, SD: 17.36, $p = 0.013$) were found. Furthermore, the trends of increasing PWV (from 11 m/s, SD: 3, to 12 m/s, SD: 3, $p = 0.095$) and decreasing CRAE (from 138.87 μm, SD: 12.19, to 136.77 μm, SD: 13.19, $p = 0.068$) were recorded.
Conclusion: The present study investigated cardiovascular changes following COVID-19 infection at two-time points after hospital discharge (baseline measurements and 60 days post-hospitalization). Significant changes were found in systolic blood pressure, heart rate, and microvasculature indicating that vascular adaptations may

Abbreviations: SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BP, Blood pressure; HR, Heart rate; PWV, Pulse wave velocity; CRAE, Central retinal artery equivalent; CRVE, Central retinal vein equivalent; AVR, Artery to vein ratio; COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

* Corresponding author at: Division of Physiology & Pathophysiology, Otto Loewi Research Center for Vascular Biology, Immunology, and Inflammation, Medical University of Graz, Neue Stiftingtalstraße 6/D.05, A-8010 Graz, Austria
E-mail address: nandu.goswami@medunigraz.at (N. Goswami).

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be ongoing even weeks after hospitalization from COVID-19 infection. Future studies could involve conducting additional interim assessments during the active infection and post-infection periods.

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus. SARS-CoV-2 enters the cell by binding its spike protein to membrane-bound angiotensin-converting enzyme 2 on the host cells (Chung et al., 2021; Lowenstein and Solomon, 2020; Invernizzi et al., 2020; Aşıkgarip et al., 2021; Aydemir et al., 2021; Invernizzi et al., 2021; Carreno et al., 2022). Post-mortem examinations have revealed that the coronavirus SARS-CoV-2 spreads through the bloodstream and attacks various organs, including the cardiovascular system (Vymazalová et al., 2023; Shankar et al., 2022). We know that the pathophysiological impacts of COVID-19 lead to a proinflammatory phenotype, disruption of oxide-redox metabolism, reduction in nitric oxide bioavailability, and many other factors leading to endothelial dysfunction. Gradually accumulating structural changes in the endothelium, supported by spreading inflammation, can induce a prothrombic phenotype and potentially lead to thrombosis (Chung et al., 2021; Lowenstein and Solomon, 2020; Wadowski et al., 2023; Salton et al., 2022). How the viral infection affects vascular health in peripheral circulation is unclear.

The peripheral vascular system consists of blood vessels (arteries, arterioles, capillaries, venules, and veins) outside the heart. Arteries are vessels under high pressure, responsible for supplying organs with blood and nutrients. The main roles of arterioles are to regulate pressure, flow, and nutrient delivery to the tissue. Their structure which includes elastic tissue and smooth muscles, predisposes them to be primary site for vasomotor regulation of blood pressure. Capillaries branch from arterioles and are followed by venules. Capillaries lack smooth muscle and elastic tissue and are responsible for transferring nutrients and oxygen to tissues. The postcapillary part of microcirculation, venules, has a thin layer of smooth muscle and elastic tissue, making them highly reactive to extravasation and inflammation (Granger and Rodrigues, 2016). When venous endothelial cells recognize the inflammatory phenotype of the blood, they initiate the inflammation process, that intimately leads to the adherence and extravasation of leukocytes into the interstitium through dilated junctions between cells.

COVID-19 can cause endothelitis (Varga et al., 2020), with a microvascular function that is not completely recovered 3 months after the infection (Tehrani and Gille-Johnson, 2021). Additionally, lower vascular function and higher arterial stiffness provide evidence of COVID-19's impact on larger arteries (Ratchford et al., 2021; Jud et al., 2021). The virus can induce cardiovascular conditions such as acute coronary syndrome, myocarditis, arrhythmias, and valvular damage (Ruzzenenti et al., 2021). These effects, along with reduced access to cardiovascular care during the pandemic, have led to increased morbidity and mortality. Further research on the connections between COVID-19 and the cardiovascular system as well as the validation of fast and appropriate diagnostic approaches to prevent the uncontrollable spread of the disease and enable early treatment are warranted.

Pulse wave velocity (PWV) is a widely used non-invasive physiological biomarker to assess changes in arterial stiffness (Vlachopoulos et al., 2010). A PWV instrument measures the transmission of the arterial wall wave velocity between two arterial points, with carotid-femoral PWV accepted as the gold standard (Laurent et al., 2006). The normal mean PWV in the general middle age population (45–65 years) is estimated at 6.0 m/s but increases with the height of the person, age, and blood pressure (van Hout et al., 2021). PWV serves as an independent predictor of cardiovascular risk. Increased arterial PWV has been reported to be a predictor of cardiovascular risk and mortality (Vlachopoulos et al., 2010). Conditions or processes affecting the elasticity of the arterial wall are reflected in PWV changes. One of the most common pathological conditions analysed and evaluated using this approach is atherosclerosis-caused arterial stiffening (Wang et al., 2015).

Retinal image analysis is a methodology to non-invasively and quickly evaluate microvascular health from static photographs of the retinal fundus. It assesses the parameters of retinal arterioles and venules. Changes in these parameters can reflect and predict future changes in larger vessels of the cardiovascular system (Hanssen et al., 2022). Therefore, implementation this method to assess early changes of the cardiovascular system could aid in the diagnosis and treatment of various cardiovascular diseases.

VICORDER® device measuring HR, BP, and PWV and retinal imaging technique to assess microcirculation were used to evaluate the effect of COVID-19 infection on vascular health. To assess the effects of COVID-19 on health, baseline measurements were conducted as soon as possible after discharge from hospitalization; either on the day of hospital discharge (if a PCR test on the day of hospital discharge was negative) or on day 10 (if a PCR test on the day of hospital discharge was positive). The second measurements were carried out 2 months after hospitalization.

The aim of this study is to investigate cardiovascular changes, including both microvascular and macrovascular parameters, in patients who have been discharged from the hospital following COVID-19 infection. Specifically, the study aims to examine these changes at two different time points post-hospital discharge, with the first measurements taken either on day of hospital discharge or on day 10 after hospitalization, while the second set of measurements was conducted 60 days post-hospitalization. By evaluating the cardiovascular changes in the post-hospitalization period, the study provides insights into the cardiovascular changes/adaptations occurring after COVID-19 infection.

2. Material and methods

The longitudinal study was conducted in the general hospital Izola, Slovenia. The data analysis was performed at the Medical University of Graz, Austria. The study was submitted to and approved by the institutional Ethics Committee of general hospital Izola with the application number 1/21 (from 1.2.2021). The clinical trial protocol was registered on ClinicalTrials.gov, with an identifier number NCT04860206. Data collection was performed in accordance with good clinical practices and followed the WMA Declaration of Helsinki (2013). Every participant received detailed information about the study protocol and provided written consent.

2.1. Participants

Forty-three patients were invited for this longitudinal study. Participants were approached by a physician who explained the study protocol. Inclusion criteria were signed informed consent and completed hospital treatment after a positive polymerase chain reaction (PCR) nose swab test on the SARS-CoV-2 virus. The exclusion criteria were a positive PCR test for the SARS-CoV-2 upon discharge from the hospital, major injuries/damage to the musculoskeletal system (disability), and the inability to follow the instructions while performing the tests. However, if only a positive PCR test for the SARS-CoV-2 upon discharge from the hospital, then participants were included and assessed ten days after hospital admission. Out of 43 initially enrolled patients, 35 completed the study. It is highly probable that the ongoing COVID-19 pandemic and the large distance between the hospital and their homes played a significant role in the decision of eight patients to withdraw from the study, as they did not provide specific reasons for their

discontinuation. At admission, 25 reported dyspnoea and 33 showed the need for oxygen based on oxygen saturation level below 90 %. No patient received non-invasive mechanical ventilation or was intubated. The patients were hospitalized for 7.0 ± 4.9 days on average ranging between 1 and 30 days. Out of these, two patients spent 8 and 21 days, respectively, in the intensive care unit.

2.2. Study protocol

Participants were tested on two points in time after discharge from hospitalization (Fig. 1). The baseline measurements (post-COVID-19, collected between the 7th of January and to 18th of March 2021) were taken on the day of hospital discharge if a negative PCR test was obtained (PCR test, done in General Hospital Isola); otherwise, they were conducted on the 10th day after hospitalization. The second measurement was done 60 days after hospitalization (collected between 11th of March and the 19th of May 2021). The aim of this study was to investigate the changes in the cardiovascular health of post-COVID-19 patients during an encouraged but not supervised recovery period. After the first measurements, participants received a brochure titled "Stay active" which described appropriate motor and cognitive exercises along with nutritional recommendations (Pišot et al., 2020). All measurements were performed in the same room in the General Hospital Isola during morning hours. Physiological measurements using the Vicorder and retinal imaging techniques were used to assess cardiovascular health. This study was part of a larger project in which other measures, such as physical performance tests, pulmonary function, muscle properties and cognitive abilities were addressed.

2.3. Cardiovascular measurements

All measurements were performed using the VICORDER® (SMT medical GmbH & Co. KG, Würzburg, Germany). The non-fasting participants were in a supine position in a quiet room, with the head raised to approximately 15° so that the skin and muscles over the carotid were relaxed. Pulse wave velocity was measured by a cuff placed over the right carotid and the right thigh. The length between the carotid and femoral arteries was measured between the suprasternal notch and the mid-point of the thigh cuff (Stoner et al., 2012). Measurements were taken until pressure waveforms over the carotid and thigh area were clear and reproducible. During the second measurement, the same length was assured. To ensure accurate pulse wave velocity measurements, blood pressure readings were obtained using the VICORDER® device with a cuff placed on the left upper arm while the person was seated. Only one measurement per person, per session was taken in accordance with the ESH/ESC guidelines.

2.4. Microvascular measurements

The optic disc-focused retinal images (resolution of 1536 × 1536) from patients were collected in two runs, as mentioned in the study protocol. The retinal images of the right eye were taken by a trained person using a hand-held, portable 30° field-of-view digital retinal camera Optomed Aurora (Optomed Oy, Oulu, Finland). The retinal images were analysed by a trained grader without any previous knowledge about the details of the study. Image processing was done using MONA REVA software (VITO, Mol, Belgium; (Khan et al., 2022)). Details about the software and its use are reported in Khan and coworkers (Khan et al., 2022). The diameters of the 6 largest arterioles and 6 largest venules were used in the revised Parr-Hubbard-Knudtson formula (Knudtson et al., 2003) for calculating the central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE). The CRAE and CRVE were expressed in micrometres (μm).

2.5. Statistical analysis

All collected data were checked for normal distribution by the Shapiro-Wilk test. The pulse pressure index (PPI) was not normally distributed and thus transformed by a decadic logarithm, and analysed separately by a non-parametric Wilcoxon test. To compare the effect of post-COVID-19 changes, paired samples *t*-test and analysis of covariance (to check the covariates: age, height, weight, and body mass index) were applied. Since there are only 2-time points, Mauchly's test of sphericity assumes an ε of 1 for all parameters. All analyses were performed by SPSS (Version 27.0, SPSS Inc., USA). Significant results ($p \leq 0.05$) were boldened.

3. Results

Thirty-five participants (30 males, 5 females; age: 60 ± 10 years; height: 174.6 ± 8.8 cm; weight: 97.1 ± 16.7 kg; and BMI: 31.9 ± 4.9) completed this study. Over time, a decrease in heart rate (HR) (p = 0.001) and CRVE (p = 0.013) and a decrease in systolic blood pressure (SBP) (p = 0.004) were observed. PWV was higher (p = 0.095) and central retinal arteriolar equivalent (CRAE) lower (p = 0.068), respectively. Table 1 summarizes the basic characteristics of the study

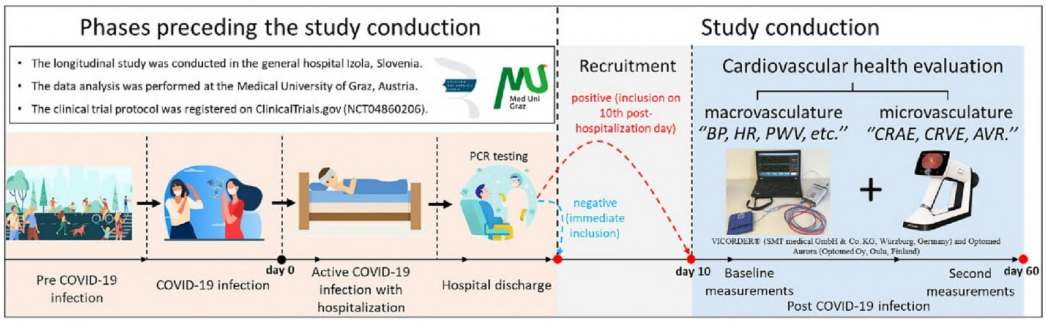

Fig. 1. Simplified overview of the study flow including phases preceding the study conduction. The vertical line with the arrows toward the right poses the time flow of the study. The black dot highlights day 0 (the first hospitalization day). The red dots highlight the points of the baseline and second measurements. The baseline measurements were conducted after discharge from hospitalization; either on the day of hospital discharge (if a PCR test on the day of hospital discharge was negative) or on day 10 (if a PCR test on the day of hospital discharge was positive). The second measurements were carried out 2 months (60 days)after hospitalization. Indicated are also the devices used to measure the parameters of (micro-) macrovasculature and measured parameters. Blood Pressure (BP), Heart Rate (HR), Pulse Wave Velocity (PWV), Central Retinal Arteriolar Equivalent (CRAE) and Central Retinal Venular Equivalent (CRVE), Artery to Vein Ratio (AVR).

Table 1
Characteristics of study participants population. The data are presented as mean (standard deviation), unless otherwise mentioned.

Parameter	Basic characteristics of the study participants
N (males/females)	35 (30/5)
Age (years)	60 (10)
Height (cm)	174.6 (8.8)
Weight (kg)	97.1 (16.7)
Body mass index (kg/m^2)	31.9 (4.9)
Dyspnea (n)	25
O2 saturation < 90 % (n)	33
Patients in intensive care unit (n)	2
Hospitalization time (days)	7 (4.9)

participants.

3.1. Cardiovascular measurements

Comparing macrocirculation parameters between two measurements after discharge from hospitalization (Table 2), two significant changes were noted. An increase in SBP ($\bar{X}_1 = 142$; $SD_1 = 15$; $\bar{X}_2 = 150$; $SD_2 = 19$; $F_{(1,32)} = 4.555$; $p = 0.041$) and a decrease in HR ($\bar{X}_1 = 76$; $SD_1 = 15$; $\bar{X}_2 = 69$; $SD_2 = 11$; $F_{(1,34)} = 14.268$; $p = 0.001$) were observed. Additionally, an increase in PWV ($\bar{X}_1 = 11$; $SD_1 = 3$; $\bar{X}_2 = 12$; $SD_2 = 3$; $F_{(1,32)} = 2.966$; $p = 0.095$) was observed.

*$\bar{X}_1$ (the mean value of the baseline measurement); $\bar{X}_2$ (the mean value of the second measurement); SD_1 (the standard deviation of the baseline measurement); SD_2 (the standard deviation of the second measurement).

3.2. Retinal microcirculation

Comparing microcirculation parameters between two measurements after discharge from hospitalization (Table 2), the present study observed a significant decrease in CRVE ($\bar{X}_1 = 204.94$; $SD_1 = 16.05$; $\bar{X}_2 = 198.05$; $SD_2 = 17.36$; $F_{(1,17)} = 7.681$; $p = 0.013$). Additionally, a reduction in CRAE ($\bar{X}1 = 138.87$; SD1 = 12.19; $\bar{X}2 = 136.77$; SD2 = 13.19; $F_{(1,17)} = 3.810$; $p = 0.068$) was observed.

*$\bar{X}_1$ (the mean value of the baseline measurements); $\bar{X}_2$ (the mean value of the second measurement); SD_1 (the standard deviation of the baseline measurement); SD_2 (the standard deviation of the second measurements).

Table 2
Comparison of the macro- and microcirculation parameters between two measuring time points after hospital discharge. Baseline measurements were conducted on the day of hospital discharge (if a PCR test on the day of hospital discharge was negative) or on day 10 (if a PCR test on the day of hospital discharge was positive) and second measurements were carried out 2 months after hospitalization. The *P*-values in bold highlight significant results. The data is presented as mean (standard deviation). SPB (systolic blood pressure), DPB (diastolic blood pressure), L (length), HR (heart rate), PWV (pulse wave velocity), PPI (pulse pressure index), CRAE (central retinal artery equivalent), CRVE (central retinal vein equivalent), AVR (artery to vein ratio).

Parameter	Baseline measurements	Two months after hospitalization	p-Value
Macrocirculation			
SBP (mmHg)	142 (15)	150 (19)	**p = 0.041**
DBP (mmHg)	76 (13)	79 (12)	$p = 0.105$
L (cm)	90 (5)	90 (4)	$p = 0.747$
HR (bpm)	76 (15)	69 (11)	**p = 0.001**
PWV (m/s)	11 (3)	12 (3)	p = 0.095
PPI	1.1 (0.3)	1.1 (0.4)	$p = 0.880$
Microcirculation			
CRAE (μm)	138.87 (12.19)	136.77 (13.19)	p = 0.068
CRVE (μm)	204.94 (16.05)	198.05 (17.36)	**p = 0.013**
AVR	0.679 (0.055)	0.693 (0.069)	$p = 0.188$

4. Discussion

The present study investigating the effect of COVID-19 infection on vascular health compared macro and microvascular parameters of the study participants at two-time points after hospital discharge. Baseline measurements were taken either on day 0 (if a PCR test on the day of hospital discharge was negative) or on day 10 (if a PCR test on the day of hospital discharge was positive), and the second measurement occurred 2 months after hospitalization. A significant increase in SBP, a lower HR, and a narrower CRVE were found (Table 2). Furthermore, there were recorded trends of increasing PWV and decreasing CRAE (Table 2).

Some studies did not find significant changes in BP between COVID-19 patients and non-infected individuals (Zanoli et al., 2022; Heckel et al., 2022; Lambadiari et al., 2021). On the other hand, the preliminary results of the LOCHINVAR study showed that COVID-19 patients have an 8.6 mmHg increase in their average 24 h SBP, compared to healthy controls (Lip et al., 2022). However, compared to the present study group, they showed elevation of SBP in actual COVID-19 patients, while the present study found an elevated SBP post-infection.

In another study, significantly higher SBP and diastolic blood pressure (DBP) in non-COVID individuals compared to COVID-19 patients were observed (Schnaubelt et al., 2021). Because the first measurements of the present study were done immediately after hospital discharge, when the effect of COVID-19 on the body is still distinct, these measurements could be considered as COVID-19 patients and the second measurements as healthy individuals. It would suggest, that the findings in the present study are in partial agreement with their findings.

There are two studies that observed long-term effect on BP post COVID-19 disease (Akpek, 2022; Szeghy et al., 2022). One includes 154 COVID-19 patients and confirmed significantly higher SBP as well as DBP post COVID-19 (31.6 ± 5.0 days after baseline) than on admission (baseline) (Akpek, 2022). Even though the duration of their study was shorter than the present study, the results of both studies are in partial agreement.

A longitudinal study, observing post-COVID-19 effect on BP showed significant reduction in SPB between 1 month and 6 months after infection (Szeghy et al., 2022). Although this study was closest to the present study in terms of duration, the present study observed the opposite effect on SBP two months after hospitalization. One hypothesis is that COVID-19 caused alterations in sympathetic activity or endothelial function leading to disturbances in blood pressure regulations (Szeghy et al., 2022). High blood pressure increases the chance to a longer and more difficult COVID-19 course with increasing hospitalization rate and deaths (Guan et al., 2020; Roncon et al., 2020).

COVID-19 may cause irregularities in HR. Ongoing inflammation and fever in the body affect the heart, causing it to pump more blood to overcome the infection. The effect can be reinforced by dehydration, anxiety, or medications, common conditions during ongoing disease. The present study showed a significant reduction in HR after COVID-19 infection (Table 2). Other studies did not disclose any significant change in HR between COVID-19 patients and the control group (Zanoli et al., 2022; Heckel et al., 2022; Lambadiari et al., 2021). Maloberti et al. found higher HR in COVID-19 patients at admission (90 ± 18 bpm) than at discharge (mean decrease of 10 bpm) (Maloberti et al., 2021). In another study where COVID-19 patients and control group were compared, the tendency in higher HR in COVID-19 patients was observed (Schnaubelt et al., 2021). This result is partially in agreement with the present study in which a reduction in HR after two months of recovery was observed.

Natarajan and colleagues presented 3-phases course of HR during

COVID-19 (1. the elevation during onset of symptoms (maximal peak), 2. decreasing to minimum around 13 days after symptoms onset, 3. increase around 28 days after symptoms onset) (Natarajan et al., 2022). They noted that HR returned to baseline levels around 112 days after symptoms onset. Because the present study did not measure HR at the onset of symptoms but only after hospital discharge, it is possible that the baseline measurements of the present study have been around the 28th day after symptoms onset. The second measurements of the present study would, in that case, reflect the results of Natarajan and colleagues when they showed a permanent course of decreasing HR from the 28th day after symptoms onset.

It is unclear why people experience changes to their HR after COVID-19. It can be due to the immune response during the COVID-19 infection, affecting the autonomic nervous system. However, other factors should be considered as COVID-19 disease affects the everyday lifestyle of sick people, lockdowns, prolonged inactivity, and spending weeks in bedrest for recovery can have a long-term impact on the cardiovascular system.

Vessel stiffening and worse PWV in COVID-19 patients has been observed (Ratchford et al., 2021; Jud et al., 2021; Lambadiari et al., 2021; Schnaubelt et al., 2021). The level of deterioration depends on the severity of the disease (Kumar et al., 2021). The present study recorded an increasing trend in PWV two months after hospitalization compared to the baseline measurements.

The study examining vascular alterations among young adults with and without SARS-CoV-19 revealed increased PWV in COVID-19 individuals (Ratchford et al., 2021). The worsening in PWV was confirmed even 4 months after overcoming COVID-19 (Lambadiari et al., 2021), which could partially agree with present results. Additionally, Schnaubelt et al. showed that COVID-19 is related not only to an enhanced PWV, but it correlated with the length of hospital stay (Schnaubelt et al., 2021). On the other hand, a study observing 24-hour hemodynamic load did not find any changes in PWV between adults with and without a history of COVID-19 (Heckel et al., 2022). Furthermore, opposite to the findings in present study, a case report of a 24-year-old woman showed improvement in PWV 6 weeks after overcoming Covid-19 (Jud et al., 2021). This is supported by the longitudinal study of Zanoli et al. showed a trend toward improvement in PWV in follow-up measurements (Zanoli et al., 2022). However, this improvement was still not at the level of healthy controls. These results were partially confirmed by another study that observed a significant reduction in PWV between 1 and 6 months post COVID-19 (Szeghy et al., 2022).

Current research confirms the deterioration of PWV reflecting arterial stiffening in COVID-19 patients as well as its improvement after recovery. The present study observed the opposite effect, thereby deterioration of PWV in the course of recovery. The results from the present study suggest that adaptive responses of the vascular system are ongoing even two months after hospitalization from the COVID-19 infection.

The first study investigating the effect of COVID-19 on retinal microcirculation, the SERPICO-19 study found that CRAE and CRVE values are higher in COVID-19 patients compared to unexposed subjects (Invernizzi et al., 2020). The present study partially confirmed these findings with a widening in retinal venules.

One study investigated 25 COVID-19 patients and 25 healthy controls and compared characteristics of retinal arteries and veins at baseline and at 4 months after remission. They confirmed findings in the SERPICO-19 study when they observed an increase in the diameters of retinal arteries and veins in COVID-19 patients during the disease (Aşıkgarip et al., 2021).

Similarly, the prospective case-control study from Turkey revealed dilatation not only in venules as observed in the present study but also in arteries as the effect of COVID-19 (Aydemir et al., 2021). Another study confirmed previous ones when they observed larger diameters of retinal arteries and venules in COVID-19 patients compare to unexposed subjects. Six months later, a favourable decrease in diameters in COVID-19 patients was shown (Invernizzi et al., 2021). However, the diameters of the vessels were still significantly narrower in severe COVID-19 patients compared to unexposed subjects (Invernizzi et al., 2021).

A study performed in Spain did not find a correlation between retinal vessel diameters and clinical outcome (Carreno et al., 2022). This study used a different setup with no control group or follow-up measurements, and the clinical assessment scale was different.

In addition to retinal fundus imaging, a recent review examines the microvascular changes in COVID-19 using nailfold capillaroscopy (Mondini et al., 2023). While both retinal imaging and nailfold video capillaroscopy provide valuable insights into microcirculation, they differ in terms of the anatomical location and specific vascular beds being examined. Retinal imaging focuses on the blood vessels within the retina, which receive their blood supply from the ophthalmic artery, the first branch of the internal carotid artery. As a result, retinal imaging is more likely to reflect changes in larger vessels of the cardiovascular system. Additionally, retinal imaging allows for the separate analysis of arterioles and venules. On the other hand, nailfold video capillaroscopy primarily assesses the capillaries in the nailfold region and is commonly used in diagnosing connective tissue diseases, particularly to identify the scleroderma-like pattern (Smith et al., 2020; Ruaro et al., 2019).

Hormonal response during inflammatory reactions leads to release of vasodilatation mediators such as histamine, bradykinin, and prostaglandins. A cytokine storm, which is a very typical consequence of COVID-19 accompanies these mediators and multiplies the pro-inflammatory effect (Chung et al., 2021; Lowenstein and Solomon, 2020; Wadowski et al., 2023; Salton et al., 2022). The vasodilatation and the increase in flow and pressure increase intravascular hydrostatic pressure and therefore extravasation through postcapillary venules into tissue. Furthermore, the virus causes disruption of the delicate balance between platelets and the vessel wall predominantly via the interplay between the factors such as activation of von Willebrand factor, coagulation cascade, and extensive neutrophil extracellular traps (NETs) formation, with histones as their components. These actions promote inflammation and play significant roles in the development of microthrombi, thrombosis, and tissue ischemia (Lowenstein and Solomon, 2020; Wadowski et al., 2023). In this study, significantly narrower retinal venules were observed two months after hospitalization from COVID-19 infection. This suggests ongoing vascular adaptations even two months after hospitalization from COVID-19 infection.

5. Limitations

Due to the limited number of enrolled subjects, the present pilot study did not include stratification based on the severity of infection, such as ICU hospitalization, thereby preventing the incorporation of clinical severity parameters into the model. Assessments before and during the infection were not performed and this situation hampers the correlation of the study data with the infection status. The inclusion of the participants in the study was confirmed by negative COVID-19 tests (in case PCR test on day 0 was positive, baseline measurements have been collected on day 10), thereby, the present study investigated the process of recovery and not the infection itself. The present study investigated cardiovascular physiology parameters after hospital discharge; measures prior to and during infection would have made the interpretation stronger. Considering the complexity experienced with COVID-19 patient interactions and difficulties with setting up studies during the pandemic period, such intense research protocols were almost impossible to set up. The study has been performed with reliable and well-accepted methods for assessing macrocirculation and microcirculation. Therefore, we are of the opinion that the study brings useful information about the possible impact of COVID-19 on the cardiovascular system. The present study does not have matched healthy control group to compare with. However, the confirmed previous COVID-19 infection and inclusion to the study as soon as possible after hospital discharge strongly suggest that the results of the baseline measurements are caused by the effect of COVID-19 infection, as well as that results

from the second measurements reflect an ongoing recovery from the infection. Even though only one grader was engaged in the evaluation of retinal images thereby the inter-rater variability factor could affect the results, we do not believe this is the case since the grader was specifically trained by the staff of MONA Reva. Due to the limited ability to perform repeated measurements on the same study participants caused by the ongoing pandemic COVID-19, only one image per session per participant was taken and thereby intra-rater variability factor could have an impact on reached results.

6. Conclusions and future directions

The evaluation of changes in cardiovascular health parameters using the Vicorder and retinal imaging technique was performed. Measurements were carried out at two time points: 1.) the baseline measurements were taken on day 0 (hospital discharge) if a negative PCR test was obtained; otherwise, they were conducted on the 10th day after hospitalization; 2.) the second measurement was done 60 days after hospitalization. Significant increase in SBP, decrease in HR, and CRVE and the trends of increasing PWV and decreasing CRAE were found. The present findings have important clinical implications, suggesting that vascular adaptations may be ongoing even weeks after the COVID-19 infection, which can contribute to improved patient management and follow-up care, as well as the development of targeted interventions to support cardiovascular health in post-COVID-19 patients. Future could involve conducting additional interim assessments during the active infection and post-infection periods, and emphasize the adequately balanced study group selection.

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CRediT authorship contribution statement

Conceptualization, B.Š., U.M., and R.P.; methodology, K.T., M.P., S. P., and N.G.; contributed to data collection, K.T., M.P. and S.P.; validation, P.D.B., B.Š., and N.G.; assessment and data processing, P.D.B., N. G., B.Š., P.M.F., B.N·N, M.G., S.P., K.T., M.P., L.S., U.M., K.S.-Z. and B.S.; formal analysis, A.S., B.Š., and R.N.; data curation, R.N., B.Š., and A.S.; writing - original draft preparation, A.S.; writing - review and editing, P. D.B., B.S., B.Š., P.M.F., B.N.N., M.G., S.P., K.T., M.P., L.S., U.M., K.S.-Z., O.Š., H.S., and N.G. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Sex Variations in Retinal Microcirculation Response to Lower Body Negative Pressure

Adam Saloň [1,2,*], Nikola Vladic [1,3], Karin Schmid-Zalaudek [1], Bianca Steuber [1], Anna Hawliczek [1], Janez Urevc [4], Andrej Bergauer [5], Vid Pivec [6], Vishwajeet Shankhwar [7] and Nandu Goswami [1,7,8,*]

1 Division of Physiology & Pathophysiology, Otto Loewi Research Center, Medical University of Graz, 8010 Graz, Austria; karin.schmid@medunigraz.at (K.S.-Z.)
2 Faculty of Health and Social Sciences, Inland Norway University of Applied Sciences, 2624 Lillehammer, Norway
3 College of Medicine, Medical University of Rijeka, 51000 Rijeka, Croatia
4 Faculty of Mechanical Engineering, University of Ljubljana, 1000 Ljubljana, Slovenia
5 LKH Südsteiermark, 18 8435 Wagna, Austria
6 Clinical Department for General and Abdominal Surgery, University Clinical Centre Maribor, 2000 Maribor, Slovenia
7 College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai P.O. Box 505055, United Arab Emirates
8 Department of Integrative Health, Alma Mater Europaea Maribor, 2000 Maribor, Slovenia
* Correspondence: adam.salon.pro@gmail.com (A.S.); nandu.goswami@medunigraz.at (N.G.)

Simple Summary: This study explored how retinal vasculature changes during central hypovolemia induced by lower body negative pressure (LBNP). LBNP is known to shift blood to the lower body and is routinely employed to assess the effects of central hypovolemia and/or to simulate the effects of hemorrhage on systems physiology. In this study, retinal imaging was carried out in participants of both sexes as they underwent LBNP. Surprisingly, no significant changes were observed in retinal blood flow between time points or across the sexes. This study is the first in this field, shedding light on retinal response during a moderate LBNP of −40 mmHg, which induces fluid shifts and evokes systematic physiological responses like those that occur during upright standing. However, further research is needed with stronger LBNP levels, including those that can induce pre-fainting (presyncope) states, to fully understand how retinal microcirculation adapts during complete cardiovascular collapse (e.g., during hypovolemic shock) and/or during severe hemorrhage.

Abstract: Introduction: Lower body negative pressure (LBNP) is routinely used to induce central hypovolemia. LBNP leads to a shift in blood to the lower extremities. While the effects of LBNP on physiological responses and large arteries have been widely reported, there is almost no literature regarding how these cephalad fluid shifts affect the microvasculature. The present study evaluated the changes in retinal microcirculation parameters induced by LBNP in both males and females. Methodology: Forty-four participants were recruited for the present study. The retinal measurements were performed at six time points during the LBNP protocol. To prevent the development of cardiovascular collapse (syncope) in the healthy participants, graded LBNP until a maximum of −40 mmHg was applied. A non-mydriatic, hand-held Optomed Aurora retinal camera was used to capture the retinal images. MONA Reva software (version 2.1.1) was used to analyze the central retinal arterial and venous diameter changes during the LBNP application. Repeated measures ANOVAs, including sex as the between-subjects factor and the grade of the LBNP as the within-subjects factor, were performed. Results: No significant changes in retinal microcirculation were observed between the evaluated time points or across the sexes. Conclusions: Graded LBNP application did not lead to changes in the retinal microvasculature across the sexes. The present study is the first in the given area that attempted to capture the changes in retinal microcirculation caused by central hypovolemia during LBNP. However, further research is needed with higher LBNP levels, including those that can induce pre-fainting (presyncope), to fully understand how retinal microcirculation adapts during complete cardiovascular collapse (e.g., during hypovolemic shock) and/or during severe hemorrhage.

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1. Introduction

Lower body negative pressure (LBNP) serves as a countermeasure against the headward fluid shift in microgravity during spaceflight [1]. It is also commonly used to assess an individual's cardiovascular stability during central hypovolemia, which occurs during prolonged standing and hemorrhage. By shifting blood from the upper to lower parts, particularly the pelvis, legs, and extravascular space, LBNP reduces the central venous pressure and venous return [2]. Reported differences in orthostatic tolerance across males and females have been documented. Goswami et al. reported that women have lower central hypovolemia tolerance [3]. Although the data regarding the lower tolerance in women is established [4,5], the reasons behind this discrepancy remain partially elusive.

While the effects of LBNP on physiological responses and large arteries have been widely reported, there is almost no literature regarding how these cephalad fluid shifts affect the microvasculature. The present study evaluated retinal microcirculation parameters, especially as previous studies have reported that changes in the small vessels of the retina precede changes in larger vessels. [6–15]. While constriction of the retinal vessels is seen in hypertension [8–10], retinal arteriolar constriction and venular dilatation have been shown to predict coronary heart disease and stroke [11–15]. Retinal imaging was used in this study, as it has emerged as a valid, rapid, and cost-effective method for early cardiovascular abnormality detection, which could potentially be used during application of LBNP or in space missions. While previous studies have linked microcirculation and sepsis [16–20], none examined LBNP. The present study attempted to fill this research gap regarding LBNP and microcirculation by exploring how LBNP levels (−10, −20, −30, and −40 mmHg) and sex (males and females) influence retinal microcirculation in young, healthy individuals. We hypothesized that variations in retinal microcirculation parameters across the protocol (baseline, different LBNP levels, and recovery) and between the sexes would be seen.

2. Methods

The present study was performed at the Division of Physiology, Medical University of Graz, Austria. Approval for the study was obtained from the Ethics committee of the Medical University of Graz, Austria (Ref: EK 25-551 ex 12/13). All the data were collected in accordance with Good Clinical Practice standards, and the study design was compliant with the Declaration of Helsinki of WMA (2013). All the participants received information about the study and provided written consent prior to participation. The signed consent forms are stored at the Division of Physiology, Medical University of Graz.

2.1. Sample Size Calculation

Using the typical retinal vascular changes from our previous studies [21,22] with an error probability (α) of 0.05 and a power ($1 - ß$) of 0.80, we estimated that the required number of participants was 20. Considering a possible dropout rate of 20%, we recruited forty-two adult participants (20 males, 22 females; age: 24.9 ± 5.9 years; BMI: 21.8 ± 25 kg/m^2). Of these, complete data sets were available for twenty-seven participants (n = 11 males; n = 16 females).

2.2. Participants

Forty-two adult participants (20 males, 22 females; age: 24.9 ± 5.9 years; BMI: 21.8 ± 25 kg/m^2) took part in this study. The protocol of the study was explained to the participants by the department staff. A simplified overview of the study flow can be seen in Figure 1. The detailed protocol of the study and the applied inclusion and exclusion criteria are described in Shankhwar et al. [23].

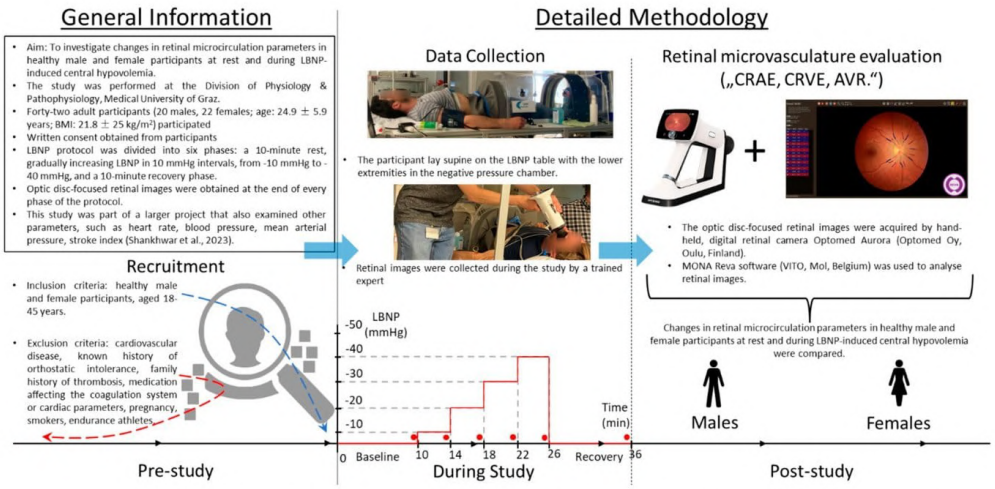

Figure 1. This diagram provides a concise overview of the study flow, encompassing the phase preceding the commencement of the study. A horizontal line accompanied by arrows as well as two blue arrows pointing rightward illustrates the study's temporal progression. The "Prior study conduction" section provides foundational study details and the recruitment specifics. The "Data collection" phase within the "Study conduction" section is portrayed through two photos captured of the study and a graph depicting temporal changes in LBNP levels. The graph charts the specific LBNP levels on the *y*-axis against minutes on the *x*-axis (including baseline and recovery periods). The red line accentuates the variation of LBNP over time. The red line accentuates the variation of LBNP over time. The red dots highlight the data collection points. The final section of the diagram introduces the retinal camera and software used for capturing and assessing the retinal images. It also emphasizes the primary study objective: examining sex-based disparities in retinal parameters during central hypovolemia. The parameters considered include the central retinal arteriolar equivalent (CRAE), the central retinal venular equivalent (CRVE), and the artery-to-vein ratio (AVR).

2.3. LBNP Protocol

The participants were measured at six-time points: at baseline supine, four times at gradually increasing negative pressures (−10 mmHg, −20 mmHg, −30 mmHg, and −40 mmHg, LBNP was increased by 10 mmHg every 4 min), and finally, during a recovery period (Figure 1). All the measurements were performed in the same room and by the same operator at the Division of Physiology, Medical University of Graz. The parameters of the microcirculation used for evaluating cardiovascular health were collected using retinal imaging. This study was part of a larger project that also examined other parameters, such as heart rate, blood pressure, mean arterial pressure, stroke index, cardiac index, total peripheral resistance index, and low-frequency and high-frequency RRI band power derived from the heart rate variability signal [23].

2.4. Microcirculation Measurements

The optic disc-focused retinal images were acquired by a hand-held, portable 30° field-of-view digital retinal camera, Optomed Aurora (Optomed Oy, Oulu, Finland), by the same trained researcher at six time points. Another trained researcher who was blinded to the study participants analyzed the retinal images using MONA REVA software (version 2.1.1,VITO, Mol, Belgium; [24]). For further details, see Saloň et al. [21]. The retinal microcirculation parameters were as follows: central retinal arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE), and artery-to-vein ratio (AVR).

2.5. Statistical Analysis

All the data were tested for normality of the distribution using the Shapiro–Wilks test, and the cases that largely deviated from the group mean and/or the missing data were excluded. Due to the small number of complete data sets, only the baseline, LBNP-40, and recovery measurements were included in the statistical analyses. Repeated measures ANOVAs were performed with sex as the between-subjects factor and three evaluated time points (baseline, LBNP-40, and recovery) as within-subjects factors to analyze changes in CRAE and CRVE. For pairwise comparisons between the different conditions (baseline, LBNP40, recovery), the significance level was adjusted according to Bonferroni. All the data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 27.0., Armonk, NY, USA: IBM Corp).

3. Results

Six (14.3%) out of the forty-two participants dropped out (did not finish the protocol) of this study. The baseline characteristics of the participants divided based on sex are shown in Table 1. Retinal images were collected from thirty-seven participants. After the exclusion of low-quality images that were unfit for analysis, complete datasets were available for $n = 27$ participants. All the results in the present manuscript were obtained by analyzing those 27 participants (Table 1).

Table 1. Baseline characteristics of the study population. Data are shown as mean ± standard deviation.

Characteristics	Females	Males
N	16	11
Age [y]	22.4 ± 2.7	24.2 ± 4.2
Weight [kg]	58.7 ± 7.3	75.1 ± 8.3
Height [cm]	167.3 ± 6.6	181.6 ± 6.9
BMI [kg/m^2]	21.0 ± 1.9	22.8 ± 2.4

Retinal Microcirculation Measurements

Table 2 displays the values of the retinal microvascular parameters for three analyzed measurement time points divided based on sex.

Table 2. The values of the retinal microvascular parameters from three analyzed measurement time points are categorized by sex. The data is presented as the mean ± standard deviation for the central retinal arteriolar equivalent (CRAE), the central retinal venular equivalent (CRVE), and the artery-to-vein ratio (AVR).

Time Point	Baseline		LBNP-40		Recovery	
Sex	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
CRAE [µm]	140.81 ± 13.26	146.90 ± 11.70	142.18 ± 10.42	149.74 ± 13.84	141.34 ± 13.54	147.41 ± 13.05
CRVE [µm]	203.97 ± 20.52	210.13 ± 23.03	205.44 ± 19.03	210.45 ± 19.39	207.77 ± 18.66	211.40 ± 20.68
AVR	0.69 ± 0.07	0.70 ± 0.07	0.71 ± 0.06	0.71 ± 0.05	0.68 ± 0.07	0.70 ± 0.06

Considering the CRAE, no significant changes between the baseline, LBNP-40, and recovery ($F(2,50) = 0.579$, $p = 0.564$) were found. Although the females showed higher values of CRAE, the effect was not significant ($F(1,25) = 2.231$, $p = 0.148$). The interaction between LBNP and sex was also found to not be significant ($F(2,50) = 0.088$, $p = 0.916$).

Like CRAE, there were no significant changes between the baseline, LBNP-40, or recovery ($F(2,50) = 0.317$, $p = 0.730$), or between the sexes ($F(1,25) = 0.485$, $p = 0.492$) in

CRVE. Moreover, no interaction was found between these two factors (F (2,50) = 0.076, $p = 0.927$). Similarly, as for the two previous variables, there were no significant changes between the baseline, LBNP-40, or recovery (F (2,50) = 0.570, $p = 0.569$), or between the sexes (F (1,25) = 0.531, $p = 0.473$) in AVR.

4. Discussion

The aim of this study was to investigate changes in retinal microcirculation parameters in healthy male and female participants at rest and during LBNP-induced central hypovolemia. This study showed no significant differences in CRAE and CRVE, either during LBNP or between male and female participants.

4.1. Retinal Microcirculation Measurements

The CRAE showed no significant changes between the baseline, LBNP40, or recovery conditions. Based on these results, the null hypothesis that the LBNP-induced fluid shift does not significantly change the CRAE cannot be rejected, because the measured parameters did not show the expected decrease in arteriolar diameter (decrease in CRAE). Exploratory analyses of the lower-grade negative pressures showed an expected decrease in CRAE from the baseline to LBNP10 and from LBNP10 to LBNP20. However, they also showed a paradoxical increase in CRAE from LBNP20 to LBNP30, reaching a maximum CRAE in LBNP40, then decreasing again in the recovery phase, reaching values close to the baseline measurements. However, none of these changes were significant. Nonetheless, the increased CRAE in LBNP40 (maximal negative pressure during this study) was an unexpected result, because one would expect reflex vasoconstriction and a decrease in CRAE. Because the effect of venule contraction in the hypovolemic state is less well established compared to the arteriolar contraction, these changes are not as unexpected as the changes in CRAE. An expected normal response in a person without cardiovascular problems (such as in our study participants, who were all healthy) would be an increased venous tone, which increases the venous return and thus the preload. This effect appears to be most relevant in the splanchnic venous system, which serves as a pool of blood that can be recruited during hypovolemia [25,26]. It appears that retinal veins may not play a significant role in maintaining a higher venous return during central hypovolemia.

One possible explanation for the non-significant changes observed in the microcirculatory blood vessel diameter could be attributed to the intricate nature of microcirculatory regulation mechanisms within the central nervous system and the retina. A study conducted by Bill et al. examined the control of the retinal blood flow, which exhibits similar flow control patterns to cerebral perfusion in healthy individuals. Additionally, the retinal microcirculation has the highest density of microvascular pericytes. However, retinal circulation lacks autonomic innervation and depends solely on local vasogenic factors like endothelin, a vasoconstrictor [27]. Similar to the blood–brain barrier, the blood–retina barrier safeguards the retinal cells from alterations in the composition of peripheral blood [27,28]. This suggests that the absence of a correlation between changes in peripheral circulation parameters, like retinal microcirculation, may be attributed to the differential control systems in operation. While the autonomic nervous system primarily regulates the peripheral circulatory system, retinal vessels are predominantly influenced by local vasogenic factors. Therefore, the lack of correlation between the retinal responses during hypovolemia with the observed systemic cardiovascular changes in the present study may be attributed to the absence of autonomic innervation and local vessel control. A study by Koep et al. (2022) further highlights significant differences in the regulation of cerebral and peripheral circulation. The authors suggest that extrapolating the regulation of cerebral vasculature based on peripheral sympathetic nerve activity is not appropriate. Contrarily, cerebral sympathetic nerve activity appears to have an opposing effect compared to peripheral circulation, and its modulation is mediated by changes in intracranial pressure and cerebral blood volume [29]. In the peripheral circulation, sympathetic nerve activity typically results in vasoconstriction of arteries and veins, leading to decreased blood flow.

Sympathetic nerve activity in the cerebral circulation can lead to both vasoconstriction and vasodilation, depending on receptor density, distribution, the presence of other vasoactive compounds, and neurotransmitter release. Additionally, the distribution and types of adrenoreceptors in cerebral vessels vary across different regions, indicating region-specific autonomic regulation of cerebral blood flow [29]. These findings reinforce the notion that the regulation of circulation is multifaceted, underscoring the complexity of cerebral blood flow regulation.

Conversely, reports of data with significant changes in the retinal microcirculation can be found; however, it must be noted that the cardiovascular stress during these studies was more drastic than that of the LBNP protocol used in this study, as most of the obtained data in those studies involved models of septic and hemorrhagic shock. A study conducted by Jurate Simkiene et al. examined changes in retinal microcirculation in patients with sepsis or septic shock compared to healthy individuals. The results showed a significantly higher CRAE during sepsis or in septic shock patients, indicating altered hemodynamic states. However, the CRVE and AVR did not differ significantly between the two groups [19]. This study aligns closely with the methods employed in our LBNP study and suggests that CRAE changes can be observed and correlated with hemodynamic alterations, particularly in septic shock. While there are similarities in the hemodynamic changes observed in septic shock and hypovolemia, it is important to note that they are not identical. In septic shock, the primary mechanism is decreased vascular tone, leading to vasodilation and impaired tissue perfusion. On the other hand, hypovolemia involves a decrease in intravascular volume accompanied by increased vascular tone, which aims to compensate for the reduced blood volume by constricting blood vessels. These distinct mechanisms highlight the different underlying pathophysiologies between septic shock and hypovolemia, despite some overlapping hemodynamic changes. Retinal fluorescein angiography performed by Erikson et al. (2017) in patients with sepsis revealed pathological retinal changes associated with arterial blood flow slowdown, such as vitreous changes, retinal hemorrhages, and fluorescein-leaking microaneurysms. Bilateral involvement was observed in 75% of the cases [20]. These findings further support the notion that retinal microcirculatory changes can be observed in hemodynamic disturbances like sepsis, although fluorescein angiography may provide better visualization compared to measuring vessel diameters. It is important to note that while our study induced a state of hypovolemia rather than a shock-like state, the mentioned studies on septic shock are relevant.

4.2. Differences in Males vs. Females

The data analysis carried out in this study did not reveal any significant differences in the retinal vessel diameters (CRAE, CRVE, or AVR) between the male and female participants during the three evaluated time points (baseline, LBNP-40, and recovery). This suggests that sex did not influence the response of retinal vessels to LBNP, contrary to our initial hypothesis. While there was a trend of higher CRAE values in the female participants throughout the study, this difference was not statistically significant. Similarly, the changes in CRAE were identical in males and females during the study protocol. We speculate that the tendency towards a higher arteriolar tone observed in males, indicated by a lower CRAE, may contribute to their enhanced orthostatic tolerance and stronger vasoconstrictor response compared to females. This notion is supported by several studies, including the work of Huxley et al., which suggests that females tend to exhibit an increase in HR rather than SVR in response to orthostatic challenges, whereas males show the opposite pattern [30]. However, it is important to note that our study did not reveal a significant difference in CRAE between males and females. Therefore, our findings do not directly confirm or refute this hypothesis. Further research specifically focusing on the relationship between retinal vessel diameters, arteriolar tone, orthostatic tolerance, and sex differences would be valuable in providing a more comprehensive understanding of these phenomena. While exploratory analyses revealed that the female participants exhibited a tendency towards higher CRVE throughout all the LBNP phases, except for the LBNP10 phase where

the male CRVE was higher, these differences did not reach statistical significance. It could be speculated that the presence of more dilated venules in females might lead to a decrease in venous return, resulting in lower preload and decreased orthostatic tolerance, which is typically more common in females. However, since our study did not demonstrate a significant difference in CRVE, we cannot substantiate this hypothesis. Regarding the AVR, it had a tendency to be higher in the female participants, likely attributed to the overall higher CRAE in the females compared to the males. Nevertheless, these differences in AVR were not statistically significant. Although no studies linking retinal AVR to changes in orthostatic tolerance could be found, a lower AVR generally correlates to a higher blood pressure and various cardiovascular risk factors (including BMI, sex, and age). AVR is usually lower in males and in the older population [31–33].

Further studies are required to gain more insight into potential sex differences in microcirculation during LBNP. Future studies could also include additional data, which could provide valuable insights into the underlying molecular biology and/or mechanisms underlying such changes. For instance, recent bulk transcriptomics studies, which could represent a strong substrate to enforce the role of previously described molecular mechanisms such as the recent studies with PMID: 36290689, PMID: 36490268, and PMID: 32184807, could be incorporated into future measurements and research.

5. Limitations

A small sample size could be considered one limitation of our study. However, we do not believe that this is the case, as we carried out a sample size calculation prior to starting the study. Due to difficulties in retinal image acquisition and analysis, complete data sets (baseline, LBNP40, and recovery) from twenty-seven out of the forty-two participants were available for final evaluation. This is in accordance with our sample size calculations, which estimated a minimum participant number of 24 to obtain 80% power.

We observed a lack of correlation in the retinal responses during hyovolemia with the observed systemic cardiovacular changes. It is possible that the lack of autonomic innervation and local control of retinal vessels could have contributed to these results. Future studies should explore this in more detail.

In addition, a maximum LBNP of -40 mmHg was used in the present study, which corresponds to the stress experienced while standing upright [3]. Several studies have used LBNP levels of ≥ -50 mmHg [4,34]. Greater negative pressures could lead to more drastic changes in retinal microcirculation parameters, which could be more similar to those seen in studies on septic and hemorrhagic shock.

Finally, the analysis of retinal images was a highly subjective process which frequently required manual adjustments, which is a potential source of error that might have influenced the results. We do not believe this is the case, as the complete data analysis was performed by the same professionally trained grader (AS).

6. Conclusions and Future Directions

The present study examined how the LBNP protocol, utilizing a maximum negative pressure of -40 mmHg, influences retinal microcirculation parameters in healthy young individuals. Graded LBNP application did not lead to changes in the retinal microvasculature across the sexes. The present study is the first in the given area that attempted to capture changes in retinal microcirculation caused by central hypovolemia during LBNP. However, further research is needed with higher LBNP levels, including those that can induce pre-fainting (presyncope), to fully understand how the retinal microcirculation adapts during complete cardiovascular collapse (e.g., during hypovolemic shock) and/or during severe hemorrhage. Additionally, future research should include retinal blood flow and velocity measures alongside vessel diameters for a more comprehensive understanding. Finally, integrating additional data in future studies can provide valuable insights into the underlying molecular biology and mechanisms driving these changes.

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Informed Consent Statement: All the participants received information about the study and provided written consent prior to participation.

Data Availability Statement: The datasets generated from the current study are available from the corresponding author upon request.

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4 DISCUSSION

This dissertation investigates alterations in retinal microcirculation across diverse populations, including both healthy individuals and patients with various medical conditions (detailed in the Aims and Hypotheses section). The dissertation work comprises a collection of twelve studies/projects, with half dedicated to healthy populations and the other half to patient groups. Given the cumulative format of this dissertation thesis, it includes exactly three first-author publications (from the twelve studies/projects within the dissertation work), along with their joint discussion.

While the impact of MLT on cardiovascular physiology was shown through narrower CRAE and CRVE, placebo therapy exhibited the opposite effect. It's important to note two key points: 1.) CRAE values after MLT returned to first/baseline measurement levels prior to the placebo; 2.) the first/baseline measurement values for the same individuals exhibited comparable variability prior to any therapy/intervention. Therefore, the observed result is likely attributed to commonly occurring fluctuations in the opposite course (increase after placebo and decrease after MLT). Additionally, there were no detected impacts on HR variability and BP variability. Notably, we found no evidence of previous research investigating the influence of light therapy on parameters of retinal microcirculation.

In the second paper incorporated into the dissertation thesis, which delved into the impact of COVID-19 on parameters of macro and microvasculature, notable findings emerged. These findings included a substantial elevation in systolic BP, a reduced HR, and a lower CRVE. Additionally, the study observed trends indicating an increase in PWV and a reduction in CRAE, shedding light on the complex cardiovascular alterations associated with COVID-19 infection.

The third and final paper included in the dissertation thesis, focusing on investigating alterations in retinal microcirculation parameters within a cohort of both male and female healthy participants, both at rest and in the context of LBNP-induced central hypovolemia, did not identify statistically significant differences in either CRAE or CRVE. These findings remained consistent, whether during the application of LBNP or when assessing responses between male and female participants.

4.1 Impact of Light Therapy on Cardiovascular Parameters and Retinal Microcirculation

Light therapy, along with yoga and meditation, belongs to the category of energy therapies, all rooted in the belief of energy fields flowing within and around the body. One study observed how cardiac rehabilitation lasting four weeks influenced two distinct groups of patients; whereas the control group received typical exercise therapy, the intervention group received in addition to typical exercise therapy transcendental meditation. The study aimed to assess reactions of cardiovascular and muscular systems (178). A significant decrease in systolic BP and a nearly significant reduction in HR have been seen. Additionally, there was a noteworthy increase in the RR interval, with no discernible differences between the two groups (178). Furthermore, our study extended the scope of this research by evaluating retinal microcirculation parameters, an additional follow-up measurement, and a third rehabilitation group, which engaged in typical exercise therapy and yoga exercises, was introduced. However, no significant results were seen during the study, as well as between the different rehabilitation groups (data not published). These conclusions bring valuable information on the potential benefits of energy-based therapies in cardiac rehabilitation, emphasizing the need for further research in this intriguing field.

The narrowing of blood vessels is known to restrict blood flow, potentially causing a BP elevation (179). Nonetheless, our investigation didn't show a significant impact of the light therapy on BP. Light therapy, particularly within the visible spectrum range, has the capacity to influence photosensitive molecules such as SODs, cytochrome C oxidase, and ATP subsequently affecting the redox homeostasis (180–182). Current research indicates that light therapy stimulates mitochondrial activity, leading to elevation in ROS and NO production with a vasodilation effect (180,183–185). Surprisingly, in our investigation, we did not find any substantial effects of light therapy on systolic and diastolic BP. The pilot study on 44 hypertensive subjects suffering from high BP, which explored the impact of laser-mediated acupuncture on BP, body mass, and HR variability, observed a reduction in systolic BP and diastolic BP. This effect followed low-level laser treatment conducted over a 90-day period with at least 12 treatments per subject (186). However, it's worth noting that our study involved a shorter duration of interventions, and consisted solely of healthy individuals, which may explain the absence of significant results in our case. To corroborate the light therapy findings, a crossover study of 14 healthy male individuals, investigated the two-day

effects of two types of blue light on cardiovascular physiology (185). The study revealed that monochromatic blue light reduced systolic BP, improved arterial stiffness, and enhanced the function of the endothelium (185). Furthermore, the noticed increase in blood flow as well as the elevation in NO species causing vasodilation contrasts with the results of our current study. Nevertheless, it's crucial to note that our study's baseline values exhibited similar variations within the same individuals even prior to any therapy/intervention, therefore the noted result may be attributed to naturally occurring fluctuations. Moreover, these findings were not substantiated by any additional effects on the investigated parameters.

In the context of HR, a study involving seven participants noted an HR reduction following 10 minutes of blue light (456 nm) exposure (187). Furthermore, another investigation into sleep showed lower HR on the night following light therapy (188). This research collected data from athletes exposed to red (660 nm) and near-infrared (850 nm) light. Each session lasted 20 minutes with a maximum of two sessions without consecutive days of therapy and a mean athlete session of 8.5 ± 7.5.

In contrast to their findings, our study revealed a minor increase in HR after light therapy ($p = 0.044$). It's noteworthy, nevertheless, that this noted effect was relatively little when the difference in the averages amounted to smaller than two beats per minute (1.616 bpm). Such a small variation could readily be attributed to minor fluctuations during the protocol. It's important to emphasize that statistics primarily reveal relative rather than absolute values, for instance, 2 or 20 bpm. Consequently, the findings necessitate cautious interpretation and merit replication in future research endeavours to establish their robustness.

A few prior studies have demonstrated that light therapy causes a HR variability reduction and induction of sympathetic activity (187,189,190). However, in our investigation, we did not detect any significant effects on parameters of BP and HR variability. Travis et al. conducted a crossover study, showing significant impacts of MLT on the subjective sense of well-being among 18 individuals with prior meditation experience (191). While the study's design paralleled ours in many aspects, it is worth noting that all participants in their study were actively practicing meditation long before attending the study. Consequently, the positive outcomes observed in their study may be mediated by the meditation effect much more than solely by MLT itself. This highlights the importance of considering participant backgrounds and the potential influence of other variables when interpreting study findings.

The previously mentioned study that noted a reduction in HR following ten minutes of blue light (456 nm) exposure, noted a concurrent HR variability reduction (187). Additionally, Yuda et al. conducted a study that demonstrated a noteworthy high-frequency (HF) reduction

and low-frequency (LF)/HF ratio elevation, indicating, that 6-minute exposure to blue, red, and green light reduces parasympathetic activity (189). The research study involving twenty participants showed that exposure to red light for 10 minutes led to an elevation in the LF/HF ratio as well as LF, signifying heightened sympathetic activity (190). Opposite effects were shown after exposure to blue light resulting in not only LF but also an LF/HF ratio decrease. These changes along with additional observing of an HF elevation, indicate greater cardiac relaxation mediated by increased parasympathetic activity. (190). These findings underscore the nuanced effects of different light wavelengths on autonomic nervous system activity.

Although we were unable to locate additional studies investigating the interconnections between light therapy and BP variability or HR variability, existing literature from yoga studies provides insights into the effects of yoga exercise on parameters of HR variability, particularly those linked to vagal tone enhancement (192–194). For instance, the study conducted by Khattab et al reported an HR variability elevation following a 5-week yoga program conducted once a week for 90 minutes, involving 11 healthy participants (7 females and 4 males, aged 26–58 years, with average age: 43 ± 11) (192). Similarly, Papp and colleagues observed an augmentation of vagal tone and sympathetic activity reduction following an 8-week yoga program (193). In alignment with these findings, a shift toward parasympathetic dominance in the sympathovagal balance was documented after a year of daily meditation practice, conducted four times per week (194). However, it's important to note that many HR variability-exploring yoga studies originate from India and often face challenges related to sample size, possessing suboptimal study quality and designs, with employing a range of heterogenous measurement techniques. (194–197). While the cumulative evidence suggests a beneficial impact of yoga on HR variability, promoting autonomic regulation toward parasympathetic predominance, further research is warranted to validate these observations.

4.2 COVID-19-Related Alterations in Blood Pressure, Heart Rate, and Retinal Microcirculation

While several studies did not show COVID-19 as a driver of BP alterations (198–200), it's noteworthy that the preliminary findings from the LOCHINVAR study indicated an increase by 8.6 mmHg in 24-hour systolic BP among COVID-19 patients compared to healthy individuals. (201). Nevertheless, it's important to highlight a distinction between our study

group and the LOCHINVAR study's findings: they demonstrated elevated systolic BP levels in individuals actively battling COVID-19, whereas our study observed elevated systolic BP levels post-infection. This divergence underscores the dynamic nature of BP fluctuations in response to COVID-19 and its aftermath.

Another study conducted by Schnaubelt et al. (2021) (202) reported COVID-19-mediated significant elevation of systolic and diastolic BP levels. Given that the initial measurements in our study were conducted directly after patients' hospital release, through the time window reflecting still abundant COVID-19 impacts, this data may be considered as ones from patients, while the subsequent data could be likened to those of healthy individuals. This interpretation would imply that the findings from our study align, at least in part, with the observations made by Schnaubelt et al. as our study observed a significant systolic BP elevation.

Two studies, conducted by Akpek (2022) (203) and Szeghy et al. (2022) (204), have investigated the COVID-19-mediated long-term impacts on BP. Akpek's one involved 154 COVID-19 patients, confirmed post-COVID-19 (31.6 ± 5.0 days after baseline) systolic BP and diastolic BP elevation (203). Despite the shorter duration of Akpek's study relative to our study, there is noteworthy partial concurrence between the findings of both investigations. Szeghy et al. (2022) reported a significant systolic BP decrease between 1 month and 6 months after infection (204). Interestingly, despite the similarity in study duration between Szeghy's research and our study, our investigation showed the reverse, when systolic BP increased a two-month post-hospitalization. As Szeghy et al. hypothesized, these discrepancies could be explained by COVID-19-mediated changes in endothelial function or by affecting the sympathovagal balance toward sympathetic activity (204). It is essential to note that high BP has been linked to a more prolonged and challenging course of COVID-19, associated with increased hospitalization rates and mortality (205,206). This underscores the significance of understanding the complex interplay between COVID-19 and BP regulation for both clinical management and patient outcomes.

COVID-19 has been associated with potential irregularities in HR. The sustained inflammation and fever accompanying the infection can impact the heart's function, necessitating an increased pumping of blood to combat the illness. This effect can be further influenced by factors such as dehydration, anxiety, or medications, which are commonly encountered with ongoing disease. Our study revealed a COVID-19-mediated significant HR decrease. However, it is important to acknowledge that several other studies (198–200) did not confirm it. Additionally, Maloberti et al. (2021) (207) observed HR elevation in

individuals infected by COVID-19 upon admission (90 ± 18 bpm) compared to their HR at discharge, signifying a mean decrease of 10 bpm. The study conducted by Schnaubelt et al. (2021) (202) identified a elevation tendency in HR in individuals infected by COVID-19. This outcome partially aligns with our study, wherein an HR decrease was observed after two months of recovery, further illustrating the complexity of HR dynamics in the aftermath of COVID-19 infection.

Natarajan and colleagues delineated a three-phase pattern of HR changes during COVID-19, comprising an initial elevation at the onset of symptoms (reaching a maximal peak), followed by a decline to a minimum approximately on the 13th day, and an increase at 28th day after symptom onset. Notably, circa 112 days after symptom onset the HR return to baseline levels was observed (208). As we did not assess HR at the beginning of symptoms, it is conceivable that our baseline data align with their 28th day after symptom onset. In this context, our second set of measurements would reflect results like those observed by Natarajan and colleagues, illustrating a sustained pattern of HR reduction following the 28th day after symptoms began. This alignment underscores the potential correspondence between the findings of our study and the HR course identified by Natarajan and colleagues.

The precise mechanisms underlying the alterations in HR following COVID-19 infection remain a subject of uncertainty. It is plausible that these changes are influenced by the immune response triggered by the infection, which may exert effects on the autonomic/vegetative nervous system. However, it is imperative to recognize that additional aspects may contribute to overall impact. The COVID-19 disease profoundly disrupts the daily lives of affected individuals, with factors such as lockdowns, extended periods of inactivity, and weeks of bedrest during the recovery phase potentially exerting lasting effects on the cardiovascular system. The interplay of several factors within the context of COVID-19 makes it challenging to pinpoint a singular cause for the observed HR fluctuations.

The literature has documented a notable stiffening of blood vessels and deteriorating pulse wave velocity (PWV) in individuals afflicted by COVID-19 (200,202,209,210). Importantly, the extent of this impairment appears to be contingent upon the disease severity (211). In consonance with these findings, our study observed a discernible, two months post-hospitalization upward tendency in PWV.

Another investigation revealed a notable elevation in PWV among young COVID-19 patients (209). Intriguingly, a persisting elevation in PWV was reported even four months following recovery from COVID-19, a finding that aligns, in part, with the results of our study (200). Furthermore, Schnaubelt et al. (2021) (202) provided evidence linking COVID-

19 to elevated PWV and its correlation with the length of hospitalization. However, in contrast to these findings, a study conducted by Heckel et al. (2022) (199), did not discern any PWV changes, when comparing COVID-19 and non-COVID-19 individuals. Additionally, contrary to our study, one case report showcased an amelioration in PWV six weeks post-COVID-19 recovery (210). This observation finds support in the study conducted by Zanoli et al. (2022) (198), indicating a tendency toward PWV amelioration, although it did not reach the levels seen in healthy controls. These findings were, to some extent, corroborated by further research, demonstrating a noteworthy PWV decrease, when compared the first and sixth months following COVID-19 recovery were compared (204).

Current studies support the notion of PWV elevation as an indicator of arterial stiffening in individuals with COVID-19, with subsequent improvement upon recovery. In contrast, our study yielded opposing results indicating a trend of PWV elevation during the recovery process. These findings from our study strongly imply that the vascular system's adaptive responses continue to evolve at least two months after COVID-19 hospitalization.

The SERPICO-19 study, which marked the inception of research interconnection between COVID-19 and retinal microcirculation parameters, reported widened diameters of both venules and arterioles in individuals with COVID-19. (212). Our current study aligns partially with these findings, as we also observed an enlargement in retinal venules, shedding further light on the evolving understanding of this phenomenon.

In a comprehensive study, comparing 25 individuals suffering from COVID-19 infection with 25 healthy controls, retinal fundus imaging was conducted both at baseline and four months post-remission. In alignment with SERPICO-19, enlargement of retinal venules and arterioles was observed among COVID-19 individuals (213).

Similar to the above, a study from Turkey showed enlargement in both, venules, and arterioles as a result of COVID-19 (214). Subsequently, another study affirmed these findings by noting COVID-19-mediated larger diameters in retinal arteries as well as venules. Additionally, a favourable reduction in vessel diameters among individuals suffering from COVID-19 was observed six months later (215). It is worth noting that even though these diameters showed improvement, severe COVID-19 patients still exhibited significantly larger vessel diameters compared to unexposed subjects (215).

A study conducted in Spain yielded contrasting results, as there was no observed correlation between retinal and clinical outcomes (216). It's important to note that this study employed a distinct methodology, lacked a control group and follow-up data collection, as well as utilized a different scale for clinical evaluation.

Nailfold capillaroscopy has also been used to investigate microvascular alterations associated with COVID-19. (217). Retinal imaging as well as nailfold video capillaroscopy are techniques used to investigate microcirculation. However, they diverge concerning the location and vascular networks they examine. Retinal imaging assesses the vascular net within the retina, supplied by the ophthalmic artery, an internal carotid artery branch, thereby this technique is inclined to capture alterations within the larger vessels of the cardiovascular system. Moreover, the retinal fundus imaging technique permits the distinct analysis of both retinal arterioles and venules. Conversely, the second mentioned technique, nailfold video capillaroscopy evaluates primarily the nailfold capillary networks and is employed in determining connective tissue disorders. (218,219).

Inflammation-mediated hormonal responses trigger production of several vasodilatory agents, such as prostaglandins, histamine, and/or bradykinin, etc. Cytokine storm, a common aftermath of COVID-19 (220–223), amplifies the pro-inflammatory effects of these agents. The elevated vasodilation increases flow and blood pressure followed by raise of hydrostatic pressure within the vasculature, facilitating postcapillary extravasation.

Additionally, COVID-19 mediates disruption of equilibrium between thrombocytes and endothelium of vessel wall, and predominantly via coactions of players like Willebrand factor, coagulation cascade, and neutrophil extracellular traps formation. The above-mentioned actions foster inflammation and are hold a crucial roles in microthrombi development and maturation, thrombosis, and tissue ischemia (221,223).

In our study, we observed significantly reduced diameter of retinal venules followed two months hospitalization caused by COVID-19. This implies that the adaptations in vasculature may persist for weeks and maybe even months post-COVID-19-hospitalization caused by COVID-19.

4.3 Sex-Related Variations in Retinal Microcirculatory Responses to LBNP Stress

This study showed, that CRAE exhibited no significant alterations among the baseline, LBNP40, or recovery conditions. Consequently, the null hypothesis suggesting that LBNP-mediated body fluid shift does not lead to a substantial change in CRAE cannot be rejected.

Exploratory analyses revealed the wanted decrease in CRAE from baseline to LBNP10 and similarly from LBNP10 to LBNP20, but not from LBNP20 to LBNP30, when a

paradoxical increase showed up, and reached its peak at LBNP40. Then, during the recovery phase reduction again took place, eventually approaching values akin to baseline. It's important to note that none of these variations reached statistical significance.

Nevertheless, the heightened CRAE at LBNP40 (maximal LBNP in study) is an outcome that was not expected. Common intuition would suggest reflex constriction of the vessels and a consequent reduction in diameter of retinal arterioles. As the interconnection between venules contraction and hypovolemia is not as well-established as the one in arterioles, the changes in CRVE are less expected.

In individuals free from cardiovascular issues (also our study population), an expected response to LBNP involves venous tone elevation, which in turn enhances preload as well as venous return. This is particularly essential within the splanchnic venous system, important reservoir of blood which is mobilized as reaction on hypovolemia (224,225). In this context, it looks like venules of the retina may not significantly contribute to sustaining central hypovolemia-mediated increased venous return.

The complex regulatory mechanisms governing microcirculation in both central nervous system as well as retina may be one plausible reason of significant result absence in retinal microcirculation. It was showed that retinal and cerebral perfusion exhibit similar blood flow control patterns in healthy individuals. Additionally, vasculature in the retina boasts the highest density of pericytes. Nonetheless, it is important to recognize that retinal circulation operates independently of autonomic innervation, relying completely on locally produced vasoactive substances (226). Similarly as the blood–brain barrier, also the blood–retina barrier acts as a guardian, shielding retinal cells from composition in peripheral blood. (226,227). This intricate framework proposes that missing correlation between changes in parameters of peripheral circulation, for instance parameters of retinal microcirculation, could be ascribed to distinct regulation systems at play.

As mentioned above, even though the autonomic nervous system predominantly governs the peripheral circulation, vessels in the retinal are chiefly subject to the influence of local vasoactive agents. Consequently, missing alignment between hypovolemia-mediated changes in retinal parameters and the alterations in systemic circulation documented in our research could be due to missing of autonomic innervation and reliance on local vaso-control of vessels. Authors of another study underscores substantial disparities in the regulation of cerebral and peripheral circulation and posit that deducing of cerebral vasculature regulation based on activity of peripheral sympathetic is an inappropriate oversimplification. It seems, that cerebral activity of sympathetic has reverse effects when compared to peripheral

circulation, and these are mediated by fluctuations in cerebral blood volume and intracranial pressure (228).

While sympathetic activity in peripheral circulation results in vasoconstriction and diminished flow of the blood, in cerebral circulation can cause either vasoconstriction or vasodilatation, and it depends on density and/or distribution of the receptors as well as other vasoactive compounds present, and finally on releasing of neurotransmitter. Furthermore, cerebral vessel adrenoreceptors types and distribution exhibit regional variations, signifying that autonomic control of cerebral blood flow is region-dependent (228). This underscores the multifaceted nature of circulatory regulation, emphasizing the intricate mechanisms governing flow of cerebral blood.

Conversely, there is research demonstrating significant alterations in retinal microcirculation. However, it's crucial to recognize that the cardiovascular stress levels in these studies were more severe compared to the LBNP protocol employed in our investigation. Most of the data obtained in these studies were derived from septic and haemorrhagic shock models. For instance, Jurate Simkiene et al. observed a notably higher CRAE during sepsis or in septic shock suggesting hemodynamic state alterations, while other retinal circulation parameters did not exhibit significant differences (229). This closely parallels the methods used in our study and implies that alterations in diameters of retinal arterioles can be observed and linked to hemodynamic fluctuations, especially in scenarios such as septic shock.

Apart from resemblances occurring in hemodynamic shifts caused by septic shock and hypovolemia, they are different. While septic shock causes vascular tone reduction, vasodilatation, and compromised perfusion of tissue, hypovolemia causes intravascular volume reduction, vascular tone elevation, and compensatory response to the diminished blood volume via vasoconstriction. These different mechanisms underscore pathophysiology differences between hypovolemia and septic shock, notwithstanding that, certain hemodynamic alterations overlap. Retinal fluorescein angiography showed retinal blood flow reduction-associated retinal pathologies, such as alterations in vitreous, haemorrhages, and microaneurysms; with 75% bilateral occurrence (230). This supports the idea of occurrence alterations in retinal microcirculation parameters during hemodynamic changes such as septic states, albeit the visualization of fluorescein angiography might be superior compared to retinal imaging technique measuring vessel diameters. It's worth emphasizing that while our study presents LBNP-induced hypovolemia rather than a septic/haemorrhagic shock-states, the quoted septic shock investigations remain, due to a gap in the literature, pertinent.

The comprehensive data analysis conducted in our research did not yield any significant disparities in the parameters of retinal vessels among the sexes across the three evaluated time points. It indicates that sex does not influence LBNP-mediated responses in retinal vessels, contrasting with our primary hypothesis. Even though there was a tendency of CRAE elevation in females during the study, this variance did not attain statistical significance ($F(1,25) = 2.231$, $p = 0.148$). The CRAE alterations over the study were identical in both sexes.

The inclination toward retinal arteriolar tone increase seen in our study in males may be a sign of their heightened orthostatic tolerance and stronger vessel constriction reaction compared to females. This hypothesis finds support in the research proposing that, while females respond by HR elevation rather than by systemic vascular resistance to orthostatic challenges, males do the reverse (231). Nevertheless, it's necessary to underscore there was no established difference on a statistical level between sexes in CRAE. Consequently, we neither confirm nor decline this hypothesis, and future investigations focusing on the interplay between retinal vascular physiology, orthostatic tolerance, and sex distinctions are warranted to contribute significantly to a complex understanding of this topic.

Our study unveiled higher CRVE values among female participants across three measured time points, however, the statistics did not reach significance ($F(1,25) = 0.485$, $p=0.492$). We can conjecture that venule dilation in females could reduce venous return, decreased preload, and diminished orthostatic tolerance, characteristics that are more prevalent in females. Nevertheless, we did not establish a difference in CRVE that would achieve significance, and therefore, we cannot confirm this hypothesis. We showed AVR increased in female participants (AVR $F(1,25) = 0.531$, $p=0.473$), which is probably primarily due to females' CRAE elevation ($F(1,25) = 2.231$, $p=0.148$), however without statistical significance. Even though there are no previous investigations interconnecting AVR and body fluid shifts, it's worth noting that AVR reduction commonly reflects BP elevation and several cardiovascular risk factors, such as BMI, sex, and age. There is the tendency of lower AVR in males and in older populations (232–234). This context serves to underscore the fact that the cardiovascular system's multifaceted nature and the relationships between retinal vessel parameters and broader physiological factors are very complex.

Additional research endeavours are warranted to enhance our understanding of potential disparities in microcirculation between genders during LBNP. Subsequent investigations might consider the inclusion of supplementary datasets, which could offer

valuable elucidation of the molecular biology and mechanisms that underlie these observed alterations.

For instance, comprehensive studies of transcriptomics serving as robust substrates for reinforcing the roles of formerly identified mechanisms of molecular biology, like those highlighted in articles with PMIDs 36290689, 36490268, and 32184807, could be seamlessly integrated into forthcoming measurements and research protocols. This integration would enable a more holistic exploration of the intricate factors influencing microcirculatory responses in different sexes during LBNP.

4.4 Summary

Several key findings related to retinal microcirculation across diverse populations and conditions have been explored. Firstly, in the investigation of light therapy's impact on cardiovascular parameters and retinal microcirculation, it was observed that CRAE and CRVE exhibited noteworthy changes. Specifically, CRAE became narrower after light therapy, while CRVE widened following placebo therapy. However, the variability in participants' baseline measurements raises the need for further confirmation of these findings, emphasizing the sensitivity of the retinal imaging approach.

Secondly, in the context of COVID-19-related alterations in BP, HR, and retinal microcirculation, favourable changes in CRVE were documented during the recovery phase. These findings shed light on the enduring adaptive responses of peripheral circulation, which persist even weeks after overcoming the infection, adding valuable insights to our understanding of the complex cardiovascular alterations associated with COVID-19.

Lastly, the preliminary results from the LBNP study did not show significant differences in parameters of retinal microcirculation. This suggests that retinal vessels may not exhibit substantial changes in response to LBNP-induced fluid shift, and the absence of significant alterations challenges the initial hypothesis. These findings prompt further exploration into the regulatory mechanisms governing retinal microcirculation and its response to central hypovolemia.

In summary, these sections collectively highlight the dynamic nature of retinal microcirculation in various physiological contexts, emphasizing the importance of additional research to validate and expand upon these intriguing findings. The sensitivity of retinal imaging techniques, the persistence of adaptive responses post-COVID-19 recovery, and the

complex interplay of factors in central hypovolemia warrant continued investigation in the field of microcirculatory research.

As highlighted in the earlier section of this thesis- *Exploring Additional Parameters in Retinal Microvasculature Quantification*, there are evident associations between the additional retinal microvasculature parameters (bifurcation angle, vascular tortuosity, the length-to-diameter ratio, and fractal analysis) and various pathologies, especially those related to cardiovascular health. Implementing these additional parameters in this dissertation thesis would strengthen the results, discussion, and conclusion sections. However, it exceeds the scope of this manuscript, limited to investigating parameters reflecting retinal vessel diameters. Therefore, future research should scrutinize these additional retinal vascular parameters to unveil their potential in evaluating cardiovascular health.

4.5 Limitations

Comprehensive info about the gems used for the light therapy and their wavelengths was unavailable, making it challenging to compare our results with former investigations, and therefore data requires careful perception. Compared to previous studies with longer light therapy durations, the length of intervention in our study was determined by MLT practitioners, and shorter exposure times might have limited the detection of cardiovascular changes. Our study focused on healthy participants, while most of the previous research on therapy as a treatment for various conditions. The light therapy effects may be more pronounced in cases of physiological imbalance. Significant effects in our study may reflect natural microcirculation fluctuations rather than the direct impact of MLT, necessitating cautious interpretation of results. The observed retinal microvasculature caliber could have been influenced by measurement errors. One person (A.S.) conducted all retinal measurements to reduce potential impact.

Limited subject enrolment precluded stratification by COVID-19 infection severity (e.g., ICU admission), hampering clinical parameter inclusion. Pre- and during-infection assessments were absent, affecting data correlation with infection status. The study focused on post-hospital discharge cardiovascular parameters, and prior/during infection measures would have enhanced interpretation. However, the study employs reliable methods for assessing macrocirculation and microcirculation, thereby providing valuable insights into COVID-19's potential cardiovascular impact. The study lacks a matched healthy control

group, but prior COVID-19 infection suggests baseline effects, with recovery effects in subsequent measurements. While a single grader evaluated retinal images, training likely minimized inter-rater variability. Due to the restrictions caused by COVID-19, one image per session per participant was feasible, potentially affecting intra-rater variability.

While the sample size in LBNP study may appear small, it aligns with our pre-study sample size calculation aiming for 80% power. The missing correlation between hypovolemia-mediated changes in retinal parameters and the alterations in systemic circulation could be due to missing autonomic innervation and reliance on local vasocontrol of vessels, which warrant further investigation. Additionally, a maximum LBNP used in our study was -40 mmHg, potentially less severe than levels in other studies, which could account for less drastic retinal microcirculation changes. Lastly, although retinal image analysis involved subjective elements and manual adjustments, these were performed consistently by a professionally trained grader (AS), minimizing potential error impact.

The decision not to include additional retinal vascular parameters (bifurcation angle, vascular tortuosity, the length-to-diameter ratio, and fractal analysis) in this dissertation thesis may limit our ability to capture comprehensive vascular changes. However, the chosen retinal parameters, specifically the diameters of retinal vasculature, align closely with the primary objective of the research community in establishing a future non-invasive marker for cardiovascular health. Moreover, the incorporation of additional metrics would have added complexity to the candidate's workload and outgrow the scope of a single dissertation project. Therefore, we strongly believe that the selected retinal parameters are sufficiently relevant to the objectives outlined in this dissertation project.

5 CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the findings from these three studies collectively offer insights into the complex interplay between various interventions and physiological parameters. The first pilot study on light therapy observed changes in CRAE and CRVE, but the significance of these changes is potentially confounded by baseline variations and the short duration of therapy. This suggests caution in interpreting these results as a direct effect of light therapy. Furthermore, the second study investigating the long-term effects of COVID-19 on cardiovascular physiology revealed significant changes in systolic BP, HR and CRVE, as well as trends in PWV, and CRAE over time, suggesting ongoing vascular adaptations even weeks after the infection. These findings have significant clinical implications for post-COVID-19 patient management and intervention strategies. Lastly, the third study focused on retinal microcirculation during central hypovolemia using LBNP, but no significant changes were observed. Future research should explore more severe hypovolemic conditions and incorporate additional measures, such as blood flow and velocity, to yield a deeper insight into the fundamental mechanisms.

Overall, these studies underscore the need for further research to unravel the intricate relationships between interventions, physiological responses, and their clinical implications. These insights can lead to more informed healthcare practices as well as targeted interventions development to support and improve cardiovascular health in various contexts.

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