Exercise and microvascular health in an ageing population:

The EXAMIN AGE study

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List of Abbreviations

3-NT	3-nitrotyrosine
ADAM	aggressive decrease of atherosclerosis modifiers
ADmax	maximal arteriolar dilatation
AFarea	arteriolar area under the flicker curve
ARIC	Atherosclerosis Risk in Communities Study
AVR	arteriolar-to-venular diameter ratio
CRF	cardiorespiratory fitness
CRAE	central retinal arteriolar equivalents
CRVE	central retinal venular equivalents
CV	cardiovascular
DVA	dynamic retinal vessel analysis
eNOS	nitric oxide synthase
EVA	early vascular ageing
EXAMIN AGE	Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Ageing Population
FID	flicker light-induced dilatation
FMD	flow-mediated dilation
HA	healthy active
HIIT	high-intensity interval training
HRmax	maximal heart rate

HS	healthy sedentary
MCT	moderate continuous training
MESA	multi-ethnic study of atherosclerosis
MET	metabolic equivalent
NO	nitric oxide
PA	physical activity
ROS	reactive oxygen species
SR	sedentary at increased CV risk
SVA	static retinal vessel analysis
VDmax	maximal venular dilatation
VFarea	venular area under the flicker curve
VO_2peak	maximal oxygen consumption
vWF	von Willebrand factor

Summary

Background

Cardiovascular (CV) disease remains a major health care burden worldwide. Exercise has a preventive effect on CV disease risk. Risk factors for CV disease include higher age, higher inactivity levels as well as reduced cardiorespiratory fitness. The retinal microcirculation is a valid vascular bed to detect vascular alterations in an early and subclinical stage. Alterations in retinal microvascular phenotype, defined as narrower central retinal arteriolar equivalents (CRAE), wider central retinal venular equivalents (CRVE) as well as reduced flicker light-induced dilatation (FID), are associated with increased CV risk. Reactive oxygen species (ROS) production, modulated by DNA methylation of $p66^{Shc}$, is a key driver for vascular alterations. To date no study exists that investigates the influence of physical activity (PA) or the effect of an exercise intervention on the ageing process of the retinal microcirculation in healthy individuals and patients with increased CV risk.

Aims

The aims of my PhD project were: 1) to investigate the association of long-term PA or inactivity on retinal microvascular phenotype in healthy older individuals, 2) to investigate the association of CV risk on retinal microvascular phenotype in long-term physical inactive older individuals and 3) to investigate the effects of twelve-weeks HIIT on retinal microvascular phenotype in older CV risk patients.

Methods

This PhD based on the “Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Ageing Population” study (EXAMIN AGE). This study investigated the exercise effects in a systems physiology approach with a cross-sectional and an interventional study design. In the cross-sectional approach 38 healthy active (HA), 36 healthy sedentary (HS) and 84 sedentary individuals at increased CV risk (SR) were included. SR were randomised into a twelve-week high-intensity interval training (HIIT) or a control condition with standard PA recommendations after the baseline assessment. The Retinal Vessel Analyser was used to

measure the retinal microvascular phenotype. Enzyme-linked immunosorbent assay kits were used to analyse plasma 3-nitrotyrosine (3-NT) as a marker of oxidative stress. Gene expression of $p66^{Shc}$ and DNA methylation analysis were assessed in mononuclear cells by real-time quantitative polymerase chain reaction and Methylminer® quantitative polymerase chain reaction to detect the epigenetic pathway of oxidative stress, one potential mechanism that affect retinal microvascular phenotype.

Results

Our results demonstrated wider CRAE and narrower CRVE in HA compared to HS resulting in a higher arteriolar-to-venular diameter ratio (AVR). By contrast, SR showed narrower CRAE and wider CRVE compared to HS resulting in a lower AVR compared to HS and HA. HS showed higher FID compared to SR and HA. FID in SR and HA did not significantly differ. A significant correlation between CRVE and maximal oxygen consumption (VO_2peak) as well as between AVR and VO_2peak were observed. In both sedentary groups, higher $p66^{Shc}$ expression and increased plasma levels of 3-NT were associated with hypomethylation of $p66^{Shc}$ promoter. HIIT reduced body mass index, fat mass, low-density lipoprotein and increased muscle mass and VO_2peak. HIIT increased CRAE, decreased CRVE and increased arteriolar FID compared to the control group. A significant association between ΔCRAE and ΔVO_2peak, ΔAVR and ΔVO_2peak as well as between Δarteriolar FID and ΔVO_2peak were observed. HIIT restored promoter methylation, blunting $p66^{Shc}$ expression and 3-NT levels.

Conclusion

Higher PA seems to be associated with favourable microvascular phenotype compared to sedentary individuals, with a further decline in sedentary individuals with increased CV risk. However, the use of FID seems to be limited in highly active individuals, eventually due to pre-dilated arterioles. Therefore, our recommendation is to combine FID with analysis of retinal vessel diameters to differentiate functional non-responders from manifest microvascular endothelial dysfunction and thereby improve individual microvascular risk stratification. Exercise treatment has the potential to counteract microvascular dysfunction in older patients at increased CV risk. Exercise-induced reprogramming of DNA methylation on $p66^{Shc}$ gene promoter may represent a putative mechanistic link whereby exercise protects against age-

related oxidative stress. Retinal vessel analysis seems to be a sensitive tool for detecting long-term PA as well as short-term exercise effects on retinal microvascular health in an ageing population.

1. Introduction

1.1 Social economic burden of CV disease

Cardiovascular (CV) disease is still the main health care burden worldwide. The World Health Organisation statistics from 2013 show that 36 million deaths or 63% of all globally occurring deaths were due to non-communicable diseases. CV disease are the main cause of death (48%) followed by cancer (21%) and chronic respiratory disease (12%)[1].

The European Commission explained in their Companion Report "State of Health in the EU 2017" that CV disease is also a main cause of mortality in the European Union[2]. Thirty-four percent of all deaths in men and 40% of all deaths in women were caused by CV disease[3]. Despite the general mortality rate falling, health expenditure is rising. Health costs are on average the biggest government expense after pensions, which poses a significant economic burden for the European countries[2]. One reason for higher health care costs is the higher life expectancy and demographic changes. The 2015 Commission-EPC Ageing Report speculated that the number of European Union citizens aged 65 or older will rise from 27.8% today, to 51.1% in 2060. This would result in only two working-age individuals per person over 65 years, compared to four working-age individuals per person over 65 years today. The future health challenges of an ageing population mean that 1) the health care systems will be under financial strain due to less working-age individuals per person over 65 years and 2) an increasing number of individuals will suffer from chronic diseases. Therefore, prevention strategies for chronic diseases are increasingly important. To date up to 80% of health care costs are spent on the treatment of non-communicable diseases, which are largely preventable. Only 3% of the European health care budgets are currently spent on prevention. Therefore, the European Commission describes improvements in disease prevention as a main health care challenge for the future[2].

Health and disease data in Switzerland mirror the situation in the world and the European Union. The leading cause of mortality in Switzerland is also CV disease, based on the latest data from 2016. From a total of 64 964 deaths, 20 712 were caused by CV disease (32%)[4]. The

general increase in the older population aggravates the health burden in Switzerland as in other countries. The number of citizens aged 65 or older will increase from 1.5 million in 2015 to 2.17 million by 2030 and 2.69 million by 2045. About 27% of all citizens in Switzerland will be 65 years or older in the year 2050[5].

To conclude, CV disease is a worldwide health care problem. Prevention strategies and timely diagnosis are essential to reduce CV mortality and financial health care burdens.

1.2 Lifestyle prevention strategies to reduce cardiovascular risk

The American Heart Association defines smoking, physical inactivity, unhealthy diet and being overweight or obesity as the main CV risk factors[6]. Improvements in these unhealthy lifestyle parameters have the power to decrease the prevalence of CV mortality by up to three-quarters[7].

1.2.1 Cardiorespiratory fitness and mortality

In this section, I will focus on cardiorespiratory fitness (CRF) as a CV risk factor because low CRF has previously been shown as the strongest risk prediction factor for all-causes of death even compared to obesity, smoking status, hypertension, high cholesterol levels or diabetes[8, 9]. CRF predicts all-cause and CV disease mortality in healthy individuals[9-11] as well as in patients with known CV disease such as obesity, type 2 diabetes, hypertension and lipid abnormalities[10, 12-18]. Meyers et al. investigated exercise capacity on a treadmill in 6 213 men with and without a history of CV disease. The overall mortality during a mean follow-up of 6.2 years was the primary endpoint. Peak exercise capacity, adjusted for age, was the strongest predictor of death in individuals both with and without CV disease. With each one metabolic equivalent (MET) increase, corresponding to 1-km/h higher running/jogging speed, there was a mortality risk reduction of twelve percent. The authors concluded that exercise capacity is a more powerful predictor of mortality among men than other established risk factors for

cardiovascular disease[19]. Laukkanen et al. found similar results. They measured maximal oxygen consumption (VO_2peak) in 2 361 men between 42-60 years and documented fatal and non-fatal cardiac events in a 13-year follow-up. Individuals with higher fitness levels had a lower risk for coronary events. Each one MET increase was associated with a 17-29% decrease of non-fatal, and 28-51% decrease of fatal cardiac events. VO_2peak together with smoking was the strongest independent and consistent risk predictor[20]. Ekelund and colleagues investigated physical fitness using treadmill exercise testing in clinically healthy men and measured death from coronary heart disease and CV disease in a follow-up period of 8.5 years. They demonstrated that each standard deviation decrease in CRF was associated with a two to five fold higher rate of coronary heart disease or CV disease mortality rate[21]. In their meta-analysis, Kodama et al. summarised several studies on CRF and coronary heart disease events, CV disease events and all-cause mortality in healthy men and women. They included 33 studies with 187 303 participants and 11 395 events in total. They found that subjects with a low CRF had a 70% and 56% increased risk for CV and all-cause mortality, respectively[11]. The authors pointed out, those least fit individuals who increased their individual CRF to the next level of fitness, would have the highest mortality benefits. Fewer mortality benefits were observed by comparing the moderate- to high-fit group[11].

This observation leads automatically to a dose-response discussion. Several studies, including some described above, found a high mortality risk in individuals with a CRF level below five METs whereas CRF levels above eight or ten METs seem to have a protective effect[11, 12, 16, 19, 22]. Kodama et al. showed that healthy individuals with CRF ≥7.9 METs had a substantially lower risk of all-cause mortality and CV events compared with healthy individuals with CRF <7.9 METs[11]. However, far more important than the discussion about the right level of CRF, is to communicate the important public health message that every increase in CRF confers substantial health benefits, especially in individuals with pre-existing low rates of CRF[23]. The high potential of exercise-based interventions to enhance CV health will be described in the following chapter.

1.2.2 Exercise-based interventions to enhance CV health

In this chapter, I would like to discuss the potential to enhance CV health from two angles: firstly by lifestyle interventions that aim to increase physical activity (PA) and therefore reduce sedentary behaviour in long-term settings, and secondly specific short-term exercise interventions.

Improvements in CRF due to an increase of PA or exercise interventions have the potential to reduce mortality by up to 44%[24]. Blair et al. examined CRF in 9 777 men at baseline and after a follow-up period of five years. Individuals who improved from unfit to fit during these five years had a reduced mortality risk of 44%[24]. Even relatively fit men with an averageVO_2peak of 42 ml/min/kg who maintained or improved their CRF during a follow-up period of six years reduced their risk of CV and all-cause mortality by up to 27% and 42%, respectively, during an eleven-year follow-up compared to individuals who showed decreased CRF[25]. This study also demonstrated that individuals who maintained or improved their fitness could lower their CV and all-cause mortality risks independent of their baseline fitness level.

Wen et al. described the influence of moderate PA on CV and all-cause mortality in an impressive way[26]. The authors allocated 416 175 individuals to one of five activity groups (inactive, low, medium, high or very high activity) based on a self-administered questionnaire for PA. Hazard ratios for mortality risk were analysed during an average follow-up of 8.05 years. The authors reported a 14% reduction in all-cause mortality among individuals exercising for 90 min. per week or 15 minutes per day in a moderate intensity (low active group), compared to the inactive group[26]. This study demonstrates the huge impact of increased daily PA on mortality risk reduction. Gregg and colleagues investigated the effect of changes in PA behaviour in older women, aged 65 years or older. Increase PA levels during a follow-up of 5.7 years, measured by questionnaire, reduced CV mortality during the next 6.7 years by up to 36% compared to individuals who stayed sedentary, independent of age, smoking, body mass index, comorbid conditions and baseline physical activity level[27]. A population-based British cohort study (n=7 735) investigated PA data of 40-59 year old men at baseline and at a follow-up 12-14 years later. All-cause mortality was analysed four years after the second investigation. Men who were inactive at baseline and then started gentle

activities had a significantly lower all-cause mortality rate compared to men who remained sedentary (45%). These results were similar for men with existing CV disease[28]. All these studies support the important public-health recommendations to increase daily PA in healthy men and women, in individuals with pre-existing CV disease and indeed for all levels of fitness. This will reduce not just CV mortality but also lower the rate of all causes of mortality.

PA as part of the daily routine has therefore obvious benefits. Additionally, short-term exercise interventions in their own right have also been shown to decrease rates of CV mortality by increasing CRF and CV health. Unfit individuals or individuals at increased CV risk especially seem to benefit from short-term exercise interventions. The meta-analysis from Lawler et al. included 34 randomised controlled trials in post-myocardial infarction patients to estimate the effect of exercise-based cardiac rehabilitation programmes on CV outcomes. Patients undergoing these programmes had a lower risk for reinfarction as well as cardiac and all-cause mortality compared to the control group. These intervention effects were independent of exercise programme duration. This means that even short exercise programmes may lead to improved long-term outcomes in CV risk patients[29]. A further meta-analysis in 63 studies with 14 486 patients following myocardial infarction, revascularisation or with a diagnosis of angina pectoris or coronary heart disease, investigated the effect of exercise-based cardiac rehabilitation compared to no-exercise control interventions. These rehabilitation programmes reduced CV mortality and the risk of hospitalisation. As well as the improved CV mortality rates, exercise rehabilitation programmes increased health related quality of life compared to the control subjects[30]. Therefore, the European Society of Cardiology, the American College of Cardiology and the American Heart Association have defined exercise-based rehabilitation programmes as a central element in cardiac rehabilitation[31-33]. However, the debate about the best exercise modalities for short-term exercise programmes is still ongoing and will be discussed in the next section.

1.2.3 Short-term exercise modalities: the best training to improve CV health

The optimal modalities of exercise programmes are being discussed in the literature by comparing the health benefits of different durations or intensities. A general distinction is made between moderate continuous training (MCT) and high-intensity interval training (HIIT). The meta-analysis of Liou et al. investigated the ability of MCT and HIIT interventions to improve VO_2peak and CV risk factors in patients with coronary artery disease. In ten studies 218 patients did a HIIT and 254 a MCT. HIIT led to a higher improvement of VO_2peak (+1.78 mL/kg/min) compared to MCT. However, MCT was associated with a more marked decrease in resting heart rate (-1.8/min) and body weight (-0.48 kg)[34]. The authors did not consider if the reduced body weight was associated with reduced lean body mass or fat mass. It is possible that HIIT leads to higher improvements in muscle mass compared to MCT thereby total body weight is not necessarily the best parameter to describe exercise-related intervention benefits. A better parameter to describe the health benefits after exercise interventions is vascular function[35]. Vascular dysfunction, especially endothelial dysfunction, occurs early in the process of atherosclerosis[36]. Patients without clinical evidence of atherosclerosis but with CV risk factors showed impaired vascular function measured by endothelial vasodilation[37]. Furthermore, several studies demonstrated that endothelial dysfunction is an independent predictor of future cardiovascular events in patients with CV risk factors and of heart failure[38, 39]. Improvements in endothelial function correlate with a more favourable prognosis[40] suggesting that vascular health, measured by endothelial function, could be a sensitive tool for detecting the effectiveness of exercise therapies. In their meta-analysis, Ramos et al. analysed the effect of MCT or HIIT interventions on vascular function in six randomised controlled trials including 182 individuals. HIIT was defined as four intervals of four minutes at 85-95% of maximal heart rate (HRmax) with three minutes of active recovery (60-70% HRmax) in between. Both exercise modalities improved vascular function. However, HIIT was more effective and additionally showed a tendency for larger improvements of VO_2peak, CV risk factors, oxidative stress, inflammation and insulin sensitivity[41]. A further meta-analysis from Weston et al. compared the efficacy of HIIT compared to MCT in ten studies with 273 patients with coronary artery disease, heart failure, hypertension, metabolic syndrome and obesity. Patients undergoing HIIT showed almost

double the increase in VO_2peak compared to MCT[42]. A broad range of publications support this theory by showing that HIIT improves CRF more than MCT in healthy individuals[43] as well as in heart failure patients[44]. As described in the previous section, CRF seems to be an important marker for CV and all-cause mortality, more so than other established CV risk factors[45], and sees greater improvement with HIIT sessions compared to MCT. Taking into account that the risk of exercise-related CV events seems to be equally low for HIIT and MCT interventions[46], and considering the fact that HIIT shows better physiological adaptations compared to MCT, it leads to the conclusion that HIIT should be preferred in short-term exercise programmes to achieve the highest improvement in CV health.

In summary, it seems to be very important to live a physically active life and increase CRF to reduce the individual CV and all-cause mortality risks. However, even if PA and fitness are strong risk predictor for CV mortality, vascular alterations and dysfunction are essentially the underlying reason why vascular events occur. To date it is not clear, which biomarker best reflects vascular alterations or dysfunction and is therefore the most sensitive biomarker for vascular ageing. The following chapter will discuss the concept of early vascular ageing as well as potential circulating and vascular biomarkers for detecting CV disease risk.

1.3 Biomarkers of CV disease

The current guidelines of the European Society of Cardiology and ten other societies on CV disease prevention in clinical practice have summarised the most important biomarkers for CV risk assessment[33]. The recommendation to evaluate CV risk is based on classical risk factors such as patients' age, smoking status, cholesterol and blood pressure. Scoring systems should be used to calculate the ten-year CV risk based on these classical risk factors. Typical scoring systems are Framingham[47], SCORE[48], or PROCAM[49]. All these systems are based on large representative cohorts and have been externally well-validated[50]. Even if these scores have their advantages, they do not measure vascular function itself and fail to measure vascular ageing directly. Nilsson et al. previously described the concept of vascular ageing or early vascular ageing (EVA) as pathophysiological model to better define individual CV risk[51].

Vascular ageing can be measured by analysing target organ damage as a mediating step between CV risk factors and CV disease events. Analysing target organ damage can enhance the screening process of CV risk patients as well as the control of treatment efficacy. Nilsson et al. described the control of risk factors in CV risk cohorts as an aggressive decrease of atherosclerosis modifiers (ADAM). ADAM have the potential to reduce target organ damage and therefore improve vascular ageing[51, 52]. Zethelius et al. demonstrated that a combination of several circulating and vascular biomarkers together with classical risk factors improved the risk stratification for CV death compared to classic risk factors solely. Circulating or vascular biomarkers, as markers for target organ damage, are likely to have a high reclassification potential[33] and will be discussed in this chapter.

Specifically vascular dysfunction, often analysed by measuring endothelial dysfunction, seems to play a key role in several disease states as a primary determinant of pathophysiology, which leads at the end to multiple organ failure and death[53-55]. Increasing endothelial dysfunction, as a marker for vascular ageing, seems to be responsible for uncontrolled clotting activation, capillary micro-thrombi formation, local hypoxia and ischaemia[53] and is associated with increased cardiac events[56]. Age-related vascular dysfunction can occur in the absence of a diagnosed CV disease and classical CV risk factors[57]. This supports the assumption that vascular dysfunction may be a precursor to the development of CV disease[58]. A key driver for endothelial dysfunction is the imbalance between nitric oxide (NO) bioavailability and reactive oxygen species (ROS)[59]. ROS generation is mediated by an upregulation of the adaptor protein $p66^{Shc}$ that is regulated primarily by DNA methylation and posttranslational modifications of histone proteins[60, 61]. Reduced $p66^{Shc}$ expression seems to protect against age-induced and ROS-mediated endothelial dysfunction, possibly contributing to the extended life span of $p66^{Shc}$ deficient mice[62]. Genetic deletion of the $p66^{Shc}$ gene has been shown to protect against age-related vascular dysfunction, most likely by reducing production of O_2^- and restoring NO bioavailability[60]. Therefore, the timely detection of endothelial dysfunction and its underlying mechanisms at an early and subclinical stage is important for primary prevention. Furthermore it is crucial to identify specific therapies that ameliorate existing endothelial dysfunction as a means of secondary prevention[55]. Normally endothelial dysfunction is not measured in the daily clinical routine[53] although there are two common ways of identifying it.

1) Evaluating an endothelial-dependent dilation response to a set stimulus or 2) evaluating circulating biomarkers of endothelial dysfunction[63].

The most frequently used circulating biomarker to measure endothelial dysfunction is the "von Willebrand factor" (vWF)[55]. This endothelial specific ligand for platelet glycoproteins plays a crucial role in platelet adhesion to damaged arterial walls[64]. vWF is released if endothelial cells are injured and can be measured by enzyme-linked immunosorbent assay. Lip and Blann demonstrated that vWF is increased in CV disease[65]. It has also been shown that vWF predicts ischaemic heart disease[66, 67] and stroke risk in previously healthy individuals[68] and is an independent predictor of acute myocardial infarction or mortality[69, 70]. Treatment of CV risk factors such as hypertension[71] or diabetes[72] results in a significant reduction of vWF. vWF seems to be a highly relevant and specific circulating biomarker for endothelial dysfunction that plays a role in the early development of several vascular diseases. Another circulating biomarker for endothelial dysfunction and endothelial cell damage are circulating endothelial cells[55]. These cells, often defined as the expression of glycoprotein CD146, are rarely found in healthy individuals but are elevated in CV[73] and inflammatory disease patients[73]. Increased levels of CD146 were associated with decreased vascular function measured by brachial artery flow-mediated dilation (FMD)[74].

The measurement of FMD is the gold standard in evaluating vascular endothelial function in the macrocirculation[75]. In this non-invasive method, vessel diameters of a target artery are measured via ultrasound 2D-images at rest, and during increased blood flow, to analyse endothelium-dependent vasodilatation of the target vessel. The underlying mechanisms for increased FMD in response to higher sheer stress were summarised previously[76]. Briefly, increased blood flow leads to higher sheer stress, which results in a calcium dependent activation of endothelial nitric oxide synthase (eNOS) and NO generation. Higher NO levels are responsible for a relaxation of smooth muscle cells and a consequent dilatation of the artery[77]. Several studies demonstrated that a reduced FMD response is associated with a higher CV event risk and highlighted the predictive value of FMD for vascular dysfunction and future CV events[78, 79]. One standard deviation increase in FMD was associated with a 50% lower risk of CV events[78]. Further means of measuring macrovascular health are the carotid artery intima-

media thickness, or the central pulse wave velocity. These are both valid biomarkers to detect vascular alterations.

However, 90% of all blood vessels and more importantly 96% of all endothelial cells are in the microcirculation, which underlines the relevance of measuring microvascular endothelial function to detect EVA. Even though the microcirculation, often defined as vessels <300µm, is a different vascular bed with its own and specific structure and function compared to the macrocirculation, evidence about a cross-talk between small and large vessels exists. Laurent et al. described the cross-talk as a vicious cycle[80]. Alterations in the microcirculation especially higher wall to lumen ratio and narrower arteriolar diameters seems to be responsible for higher blood pressure. Higher blood pressure over long period, in turn, increases the stiffness of the macrocirculation due to a shift from elastin to collagen in the vessel walls. The increased stiffness of large arteries is a major determinant of increased pulse pressure, which in turn, damages the microcirculation and favours the development of macrovascular alterations[80, 81]. Amelioration of microvascular alterations seems to be an effective approach to reduce end-organ damage by breaking this vicious cycle and therefore preserving CV health[82]. Instead of distinguishing macro- and microcirculation by their diameters, it is also possible to define these different vascular beds according to their function. This physiological definition includes all vessels in the microcirculation which show a myogenic reaction (constriction) in their diameters due to an increase blood pressure[83]. Independent of the definition, the microcirculation is the key driver in regulating the blood flow and blood pressure by changing the vessel diameter[84]. The arteriolar section is responsible for the main flow resistance and pressure decrease of the coronary tree[83]. Additionally, sheer stress, pressure and metabolic sensitivity increases with decreasing vessel diameter[85]. Therefore, it seems to be essential to evaluate vascular health and endothelial function in the microcirculation.

1.3.1 Microvascular biomarkers of CV disease

Microvascular alterations or dysfunction is often assessed by measuring microvascular endothelial function as described previously[86, 87]. Houben et al. reviewed different techniques

of assessing it[88]. The skin is a non-invasive vascular bed and can be used to analyse microvascular function with capillary microscopy or Laser-Doppler flowmetry. Capillary microscopy measures the structure and function of capillaries by evaluating capillary morphology and density, blood flow and pressure[88]. Microvascular dysfunction measured with capillary microscopy has been associated with hypertension[89], type 2 diabetes[90, 91] or obesity[92]. Laser-Doppler flowmetry measures changes in blood flow in a single spot or a larger skin area to evaluate flowmotion or blood flow after a heat or occlusion stimulus. Reduced flowmotion has been associated with higher age, and higher waist circumference as well as hypertension[93, 94]. Sörensen et al. showed a reduced heat-induced vasodilation response in pre-diabetes individuals compared to healthy controls with a further decline in type 2 diabetes patients in a population-based study[95]. To conclude, the skin seems to be a sensitive and physiologically relevant vascular bed for detecting microvascular dysfunction. However, these techniques do not allow the examination of single arteriolar or venular vessels separately. Retinal vessel analysis allows structural and functional investigation of single arteriolar or venular vessels, as well as the analysis of the whole background area of the eye. The potential of retinal vessel analysis will be summarised in the next chapter.

1.3.2 Retinal vessel analysis in CV disease

The retinal microcirculation shares its embryological origin and morphological as well as physiological properties with the cerebral circulation and is considered as a marker of cerebrovascular disease. The static retinal vessel analysis (SVA) measures arteriolar and venular diameter equivalents by analysing valid images of the eye background as previously described[96]. This technique enhances the accuracy of CV diagnosis by up to 21% and by 10.1% for CV events[97] and stroke[98]. Narrower retinal arterioles and wider venules have been associated with increased CV events such as stroke[99], coronary heart disease[100] as well as higher CV mortality[101] in an ageing population. Additionally, static retinal vessel alterations are associated with hypertension[102, 103], diabetes, obesity, dyslipidaemia and inflammation[100, 104]. Although retinal vessel diameters are key regulators of microvascular blood flow, they do not reflect microvascular endothelial function per se. Dynamic retinal vessel analysis (DVA) is

a new diagnostic tool for the assessment of microvascular endothelial function by flicker light-induced retinal vessel dilatation (FID) over time[105]. Reduced dilatation responses were found in pre-diabetes, with a further reduction in type 2 diabetes patients compared to non-diabetics with normal glucose levels[95]. Nägele et al. showed a reduced retinal dilatation response in patients with increased CV risks with a further decline in chronic heart failure patients[106]. Machalinska and colleagues showed that a reduced dilatation response is associated with circulating markers reflecting endothelial dysfunction in hypertensive patients[107]. Furthermore, a reduced FID is associated with higher age[108, 109] and obesity[110]. An impaired retinal vessel dilatation could be an early indicator, and seems to predict mortality in high-risk cohorts[111]. To conclude, the retinal microvascular phenotype, defined as central retinal arteriolar (CRAE) and venular (CRVE) equivalents and arteriolar and venular FID, seems to be a non-invasive and sensitive vascular biomarker to investigate microvascular health and subclinical vascular remodelling. The retinal microvasculature has previously been described as a window to the heart[112]. Retinal vessel analysis may proof to be a valid diagnostic tool to screen early vascular ageing (EVA) in the microcirculation. However, it remains to be elucidated whether exercise can reverse or postpone further progression of advanced vascular ageing even in older adults as a means of an aggressive decrease of atherosclerosis modifiers (ADAM).

1.3.3 Retinal vessel phenotype, physical activity and fitness

Less is known about the association of PA and fitness on the retinal microcirculation. The Atherosclerosis Risk in Communities (ARIC) Study showed that higher levels of PA during sport or at work were associated with narrower CRVE[113]. Anuradha et al. showed that lower levels of PA and higher levels of sedentary behaviour, measured with television viewing time, were associated with wider CRVE[114]. Higher CRF in childhood was associated with wider CRAE and narrower CRVE[115]. A structured daily PA programme during school breaks for eight weeks led to wider CRAE, which is associated with better CV health[116]. Hanssen et al. did the first intervention study on retinal vessel analysis in adults. The authors demonstrated that obese athletes, lean amateur athletes as well as elite athletes showed increased arteriolar-to-

venular diameter ratio (AVR) after ten weeks endurance training. Additionally, the authors found a significant and positive association between individual fitness levels and AVR[96].

The beneficial effect of high PA levels and higher CRF on the retinal microcirculation had been shown previously. However, no study currently exists that has investigated the protective effect of long-term PA or the reversible short-term effect of exercise therapy on the retinal microvascular phenotype in older adults. Therefore, our research questions were as follows:

1. PA and normal vascular ageing: Do long-term healthy active older adults have wider retinal arteriolar and narrower venular diameters as well as higher FID as compared to their healthy sedentary peers?
2. CV risk and vascular ageing: Do older adults with increased CV risk have narrower retinal arteriolar and wider venular diameters as well as reduced FID as compared to their healthy sedentary peers?
3. Exercise intervention and vascular remodeling: Does a twelve-week HIIT improve retinal microvascular phenotype in older adults with increased CV risk?

1.4 Aims and Hypothesis

Aims:

Aim 1: To compare the retinal microvascular phenotype of long-term healthy active older adults with the retinal microvascular phenotype of healthy sedentary peers.

Aim 2: To compare the retinal microvascular phenotype of older adults with increased CV risk with the retinal microvascular phenotype of healthy sedentary peers.

Aim 3: To investigate the effects of a twelve-week HIIT on retinal microvascular phenotype in older adults with increased CV risk compared to a control group with standard physical activity recommendations.

Hypothesis:

Hypothesis 1: Healthy older adults with high levels of PA and fitness have wider retinal arterioles and narrower venules as well as higher FID compared to healthy sedentary peers.

Hypothesis 2: Sedentary older adults with increased CV risk have narrower retinal arterioles and wider venules as well as reduced FID compared to healthy sedentary peers.

Hypothesis 3: Twelve-weeks of HIIT increases central retinal arteriolar diameters, decreases central retinal venular diameters and improves FID in older adults with increased CV risk compared to a control group with standard physical activity recommendations.

2. Publication 1: Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population: The EXAMIN AGE Study

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Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population: The ExAMIN AGE Study

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Background: Age is a key determinant for the development of cardiovascular disease and higher age coincides with an increased prevalence of obesity and physical inactivity. The study examines the influence of physical activity on aging processes of physiological systems focusing on the mechanisms of vascular aging.

Methods/Design: The study consists of two parts. The cross-sectional approach aims at examining the association of physical fitness and cardiovascular risk with large and small artery function in healthy older active (HOA, $n = 40$) and sedentary (HOS, $n = 40$) persons as well as older sedentary individuals with increased cardiovascular risk (OSR, $n = 80$) aged 50–80 years. In the interventional approach, the OSR group is randomized into a 12-week walking-based high intensity interval training (HIIT) group or a control condition, aiming at examining the effects of HIIT on arterial function in diseased older adults. Active lifestyle is defined as >9 metabolic equivalent of task (MET) per week and sedentary as ≤3 MET/week. Inclusion criteria for OSR are overweight or obesity (body mass index ≥30 kg/m^2) plus at least one additional cardiovascular risk factor. The primary outcome is arterial stiffness as determined by aortic pulse wave velocity (PWV). The secondary outcomes are retinal arterial and venous diameters. Further cardiovascular assessments include peripheral PWV, central haemodynamics, retinal endothelial function, carotid intima media thickness, cardiac strain and diastolic function as well as autonomic function and inflammation. Physical fitness is measured by a treadmill-based spiroergometry to determine peak oxygen uptake.

Discussion: The aim of the study is to demonstrate the importance of and need for specific physical activity programs for seniors to achieve healthier aging as a long-term goal. Vascular function defines disease- and age-related end organ damage and represents the potential to contain health at older age. This research will identify cardiovascular biomarkers that best resemble underlying cardiovascular risk in age and disease. The integrated approach will help define new recommendations for treatment guidance of exercise therapy in an aging population.

ClinicalTrials. gov: NCT02796976; registered 02 June 2016 (retrospectively registered).

Keywords: aging, vascular function, exercise, arterial stiffness, retinal vessels

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BACKGROUND

Atherosclerotic cardiovascular disease (CVD) is a chronic inflammatory disease of the circulatory system and it is a main health care threat in western countries. Almost every third death can be attributed to CVD, amounting to a total of 17.5 million associated deaths worldwide (World Health Organization, 2014). About 80% of CVD deaths are thought to be associated with arterial disorders (Thom et al., 2006). Age is one of the major risk factors for the development of CVD and demographic change further aggravates the enormous socio-economic health care challenge. The number of persons aged 60 or older will double until the year 2050, which will further aggravate the burden of age-related diseases such as CVD (World Population Prospects, 2015). Aging is associated with complex structural and functional alterations of the vascular bed (Ferrari et al., 2003).

The rise of the obesity epidemic in the last decades is another main underlying reason for the high prevalence of CVD. To date, almost 70% of adults are classified as either overweight or obese as compared to 40% 40 years ago (Lavie et al., 2009). The prevalence of obesity and the metabolic syndrome increases with older age (Ford et al., 2004). Physical inactivity is a main risk factor not only for the development of obesity but also for non-communicable diseases in general and CVD specifically. The World Health Organization (WHO) has stated that more than three-quarters of all CVD mortality may be prevented by appropriate changes in lifestyle. Blair et al. found that individuals who improved from unfit to fit over a mean follow-up of 5 years showed a reduction in mortality risk of 44% compared to those who remained unfit (Blair et al., 1995). In a large prospective cohort, Wen et al. reported a 14% reduction of all-cause mortality in individuals exercising for 90 min per week or 15 min per day compared to an inactive group (Wen et al., 2011). Vigorous-intensity exercise (>8.5 MET) seemed to yield greater health benefits in terms of all-cause mortality reduction than moderate-intensity exercise (4–6 MET).

High intensity interval training (HIIT) has been suggested to be an effective training modality for secondary prevention of CVD in older adults and seems to be superior to well-established moderate continuous exercise training with respect to improving not only cardio respiratory fitness but also the cardiovascular risk profile (Helgerud et al., 2007; Wisloff et al., 2007; Tjonna et al., 2008; Guimarães et al., 2010; Kessler et al., 2012; Molmen et al., 2012). The risk of CVD events is considered to be equally low for both HIIT and moderate continuous training (MCT) intervention strategies (Rognmo et al., 2012). Exercise training and regular PA are able to reduce the main underlying mechanisms for the development and progression of CVD such as inflammation, oxidative stress and endothelial dysfunction. However, it is still unclear which biomarker is most suitable to detect the process of vascular aging and can sensitively quantify and monitor treatment effects at older age. Novel approaches for cardiovascular risk screening and exercise treatment strategies are indispensable to counteract the growing socio-economic burden and health hazard of cardiovascular disease in an aging population.

Abbreviations: HOA, healthy older Active; HOS, healthy older sedentary; OSR, older sedentary at risk; HIIT, high intensity interval training; PA, physical activity; VO_2max, maximal oxygen uptake as a measure of physical fitness; MET, metabolic equivalent; CVD, cardiovascular disease; PWV, pulse wave velocity; cfPWV, carotis-femoral PWV; ftPWV, femoral tibial PWV; AIx, augmentation index; AIx@75, AIx at heart rate 75/min; cPP, central pulse pressure; IMT, intima media thickness; HRV, heart rate variability; SVA, static retinal vessel analysis; DVA, dynamic retinal vessel analysis; CRAE, central retinal equivalent; CRVE, central retinal equivalent; AVR, arteriolar-to-venular diameter ratio.

CARDIOVASCULAR HEALTH AND AGING: A SYSTEMS PHYSIOLOGY APPROACH

Vascular aging is a gradual process of the circulation that is aggravated by the development of cardiovascular risk factors and affects both the macro- and microcirculation (Nichols et al., 2011). Since aging is the main denominator for chronic CVD manifestations, the concept of vascular aging has been proposed to improve clinical guidance of patients with increased cardiovascular risk (Nilsson et al., 2009, 2013). The concept implies that age-related clinical or subclinical manifestations are associated with vascular alterations, which can be quantified by sensitive non-invasive vascular assessments. Consistent evidence suggests that arterial stiffness is a subclinical, strong and valid vascular biomarker for the quantification of atherosclerosis and grave cardiovascular dysfunction (Salomaa et al., 1995; Laurent et al., 2006; Vlachopoulos et al., 2010a). Arterial stiffness and the impairment of the buffer capacity of large arteries lead to elevated left ventricular afterload and left ventricular hypertrophy and, at later stages, to heart failure, worsening of coronary artery disease and increased risk of stroke (Hamilton et al., 2007). Aortic pulse wave velocity (PWV), acknowledged as the "gold-standard" method for measuring arterial stiffness, is an independent predictor for cardiovascular morbidity and mortality in the general population, elderly subjects and in patients with cardiovascular disease (Laurent et al., 2001; Sutton-Tyrrell et al., 2005; Hansen et al., 2006; Mattace-Raso et al., 2006). An increase of aortic PWV by 1 m/s has been reported to represent a risk increase of 15% in total cardiovascular and all-cause mortality (Vlachopoulos et al., 2010a). In the Baltimore Longitudinal Study of Aging, aortic PWV increased twofold across the age span and higher fitness were associated with reduced arterial stiffness in a predominantly sedentary population as well as in endurance trained older men compared to less active peers (Vaitkevicius et al., 1993). In a population-based study of 373 younger subjects in the Netherlands, it was found that the effect of habitual PA on arterial stiffness depends on its intensity and differs depending on the arterial tree segment. Vigorous but not light-to-moderate habitual PA provides favorable associations with peripheral arterial stiffness in young adults (van de Laar et al., 2011). In our study, different approaches of measuring arterial stiffness are applied in various vascular beds. Central and peripheral PWV measurements are performed as well as 24-h monitoring of central haemodynamics such as augmentation index (AIx) and pulse pressure (PP). Assessment of the macrocirculation includes the structural analysis of the carotid intima-media thickness (IMT).

It is generally assumed that increased central PWV contributes to the pathogenesis of small vessel disease, particularly the myocardial and cerebral microcirculation. Increased arterial stiffness seems to expose small vessels to highly pulsatile pressure and flow, thereby inducing damage to the microvascular bed (O'Rourke and Safar, 2005). A cross-talk between large and small arteries exists, promoting a vicious circle of increases in peripheral vascular resistance, blood pressure and arterial stiffness, eventually leading to the manifestation of micro- and macrovascular target organ damage (Laurent et al., 2009). Retinal vessel analysis is a non-invasive technique that allows the examination of the retinal microcirculation (Liew et al., 2008). Retinal vessels are part of the cerebrovascular bed and they are affected early in the process of cardiovascular disease. Large cohort studies have previously shown that narrower retinal arterioles, wider retinal venules and a resulting lower arteriolar-to-venular diameter ratio (AVR) are associated with increased risk and severity of hypertension (Wang et al., 2003; Wong et al., 2004a; Ikram et al., 2006b), risk of stroke (Ikram et al., 2006a; McGeechan et al., 2009) and a higher cardiovascular morbidity and mortality in older subjects (Wong et al., 2002; Wang et al., 2007). In older adults, obesity is associated with retinal venular widening and a lower arteriolar-to-venular diameter ratio (AVR) (Nguyen and Wong, 2006; Wang et al., 2006). Inflammation has been associated with wider retinal venular diameters (Klein et al., 2006). We have previously shown that higher physical fitness levels are associated with higher retinal AVR and that regular endurance exercise induced arteriolar dilatation as well as venular constriction, leading to a significantly improved AVR in middle-aged lean and obese individuals (Hanssen et al., 2011). Retinal microvascular endothelial function can directly be measured *in vivo* by dynamic retinal vessel imaging inducing neurovascular coupling by flicker stimulus (Falsini et al., 2002; Polak et al., 2002). An impaired response to flicker light has been associated with type 2 diabetes mellitus (Mandecka et al., 2007; Sörensen et al., 2016), high blood pressure (Nagel et al., 2004) and aging (Pemp et al., 2009; Kotliar et al., 2011). The assessment of retinal vessel diameters is used to define microvascular aging at older age and to examine associations with physical fitness. Retinal vessel analysis allows for the investigation of the cross-talk between large and small arteries.

Large artery stiffness is not only associated with small vessel disease but is known to relate to left ventricular hypertrophy as well as systolic and diastolic dysfunction (Roman et al., 2000; Weber et al., 2008; Namba et al., 2016). In patients with normal ejection fraction, increased pulse wave reflection and PWV are associated with higher left ventricular filling pressures (Weber et al., 2008). In patients with asymptomatic diastolic dysfunction, increased PWV has been shown to precede the onset of manifest heart failure with preserved ejection fraction (Karagodin et al., 2017). Indices of systolic and diastolic function share its predictive character with arterial stiffening (Vlachopoulos et al., 2010b; Kane et al., 2011; Lam et al., 2011). This suggests that ventricular-vascular interactions play a pivotal role in the clinical relevance of arterial stiffness (Chung et al., 2010). The recent Health ABC Study, however, demonstrated that the association of arterial stiffness with the development of heart failure is not independent of cardiovascular risk factors (Pandey et al., 2017). Additional assessment of left ventricular structure and function allows for analysis of the cardiovascular cross-talk and its association with cardiovascular risk factors.

Autonomic function (AF) is a principal regulator of vascular properties and cardiac function. AF alters with age and plays a crucial role in the development of cardiovascular diseases (1996; Fisher et al., 2009). Its sympathetic and parasympathetic branches regulate vascular tone and, thereby, modulate arterial stiffness (Perkins et al., 2006). Heart Rate Variability (HRV) is an easily recordable clinical marker for autonomic function and its indices have shown predictive value for the development of cardiovascular disease (1996; Kleiger et al., 2005). Increased physical activity has been shown to be associated with favorable autonomic function in older adults (Soares-Miranda et al., 2014). In addition to the above mentioned physiological systems, we will measure circulating anti- as well as pro-inflammatory cytokines and analyse their role in mediating physical inactivity- and obesity-related vascular and systemic impairments. Vascular aging and immunosenescence are explained in large part by an imbalance between inflammatory and anti-inflammatory processes. These result in a predominantly pro-inflammatory status that has been termed "inflamm aging" (Franceschi et al., 2007). Healthy aging and longevity are the result of an efficient lifelong anti-inflammatory activity that, once it fails to overcome cellular and systemic inflammatory processes, can be the driving force for frailty and age-related pathologies.

METHODS/DESIGN

Objectives

The aims of the study are twofold. In our cross-sectional research approach, the association of physical activity and fitness on the process of normal (healthy) aging is analyzed by comparing the group of healthy older sedentary (HOS) with healthy older active (HOA) adults. The association of CVD on the process of aging is examined by comparing HOS with older sedentary persons with increased cardiovascular risk (OSR). In the interventional approach, the reversibility of advanced vascular and systemic aging by a walking-based HIIT is examined in OSR.

Cross-Sectional (Part I)

Aim 1: To determine the associations of physical fitness and cardiovascular disease with large and small artery function in HOA, HOS as well as OSR group.

Interventional (Part II)

Aim 2: To examine the effects of HIIT on large and small artery function in OSR.

Outcome Measures

Primary outcome: central (carotid-femoral) pulse wave velocity (cfPWV)

Secondary outcomes: central retinal arteriolar (CRAE) and venular (CRVE) diameters

Further outcomes: peripheral (femoral-posterior tibial) pulse wave velocity (ftPWV); central augmentation index (cAIx); central pulse pressure (cPP); 24 h AIx (24AIx) and cPP (24cPP); carotid intima media thickness (IMT); retinal vessel flicker response (DVA); left ventricular diastolic function and myocardial strain; heart rate variability (HRV), inflammation.

Hypotheses

Hypothesis 1 (Part I): cfPWV is lower in HOA compared to HOS
Hypothesis 2 (Part I): cfPWV is higher in OSR compared to HOS
Hypothesis 3 (Part II): cfPWV in OSR can be reduced by 12 weeks of HIIT compared to controls.
The same hypotheses will be addressed for the secondary and further outcomes.

Study Design

The study design consists of two parts with separate sample size considerations for the cross-sectional and interventional approach. HOA and HOS participants as well as OSR are recruited and enrolled in the cross-sectional study (**Figure 1**). Recruitment of the OSR group is performed on the basis of agreement to take part in the exercise intervention following the cross-sectional assessment, which serves as a baseline examination for the consecutive intervention. Before enrolment, subjects are medically examined and scanned for inclusion and exclusion criteria by the study physician. Written informed consent is obtained from eligible subjects and a medical examination and anthropometry are performed and physical activity levels are assessed. In addition, blood sampling and 24 h blood pressure monitoring including central PWV, Aix, and PP are undertaken (visit 1). The enrolment decision is taken on the basis of the inclusion and exclusion criteria. On two separate visits and in randomized order, the main vascular diagnostics, echocardiography, autonomic function and the assessment of endurance performance (VO2max) are performed for the cross-sectional part of the study. All vascular diagnostics take place in the morning and under fasting conditions (visit 2 or 3). The endurance performance and the autonomic function is always performed in the afternoon (visit 2 or 3) to reduce within-day variability. Visit 1 and visit 2/3 are separated by at least 1 week to check inclusion/exclusion criteria. Visit 2 and 3 are separated by at least 1 day or no more than 2 weeks. After these three visits, the OSR group is randomized to take part in a 12-week HIIT or a control condition with general lifestyle recommendations. All participants have an equal likelihood of being assigned to treatment or control group. All assessments of the baseline examination are repeated in the follow-up after 12 weeks (**Figure 1**).

Inclusion Criteria

- Healthy men and women aged 50–80 years with:
  - active lifestyle: >9 MET/week (>3 h moderate walking/week) or
  - sedentary lifestyle: ≤3 MET/week (≤1 h moderate walking/week)
  - $18.5 \leq \text{BMI} < 25.0\ \text{kg/m}^2$
- Sedentary men and women aged 50–80 years with increased cardiovascular risk:
  - overweight or obesity ($\text{BMI} \geq 30\ \text{kg/m}^2$) and
  - ≥one additional cardiovascular risk factor as described in **Figure 2**.

Exclusion Criteria

- Healthy men and women aged 50–80 years with:

History of cardiovascular, pulmonary or chronic inflammatory disease; blood pressure ≥140/90 mmHg during 24 h monitoring or any of the risk factors in **Figure 2**; current or past smoker; macular degeneration or glaucoma.

- Sedentary men and women aged 50–80 years at risk:

Decompensated cardiovascular, pulmonary or chronic inflammatory disease; macular degeneration or glaucoma; compromising orthopedic problems.

Setting

The RCT will be realized at the Department of Sport, Exercise and Health, Basel, Switzerland. This study is financially supported by the Swiss National Science Foundation (SNF) and approved by the Ethics Committee of Northwestern and Central Switzerland (EKNZ-2015-351). We registered this study on ClinicalTrials.gov (NCT02796976) in June 2016.

Study Procedures and Ethical Considerations

All participants are briefed verbally and receive information approved by the Ethics Committee of Northwestern and Central Switzerland (EKNZ) giving details on the study procedures, the RCT and the process of randomization. The study will be carried out in accordance to the protocol and with the principles stated in the Declaration of Helsinki and the Guidelines of Good Clinical Practice (GCP) (World Medical Association, 2013). This study protocol was design according the SPIRIT Guidelines.

All measurements and procedures applied in this study are non-invasive. Routine exercise ECG includes an exertional stress test as a medical necessity and safety precaution to evaluate cardiorespiratory health in all subjects. The OSR group undergoes a high intensity walking-based training program. HIIT has previously been applied in a wide range of patients including patients with coronary artery disease. The risk for cardiovascular events has been proven to be equally low for both HIIT and moderate continuous intervention strategies (Rognmo et al., 2012). Retinal vessel analysis includes mydriasis of one eye. Using a mydriaticum (Tropicamid 0.5%), the pupils are dilated and enable retinal vessel analysis. The eye drops can cause temporary discomfort, oftentimes a burning sensation for 1–2 min. Flickerlight exposure can potentially cause slight headaches. The collaborating ophthalmology department in Basel will offer back up at all times in the unlikely event of continuous discomfort.

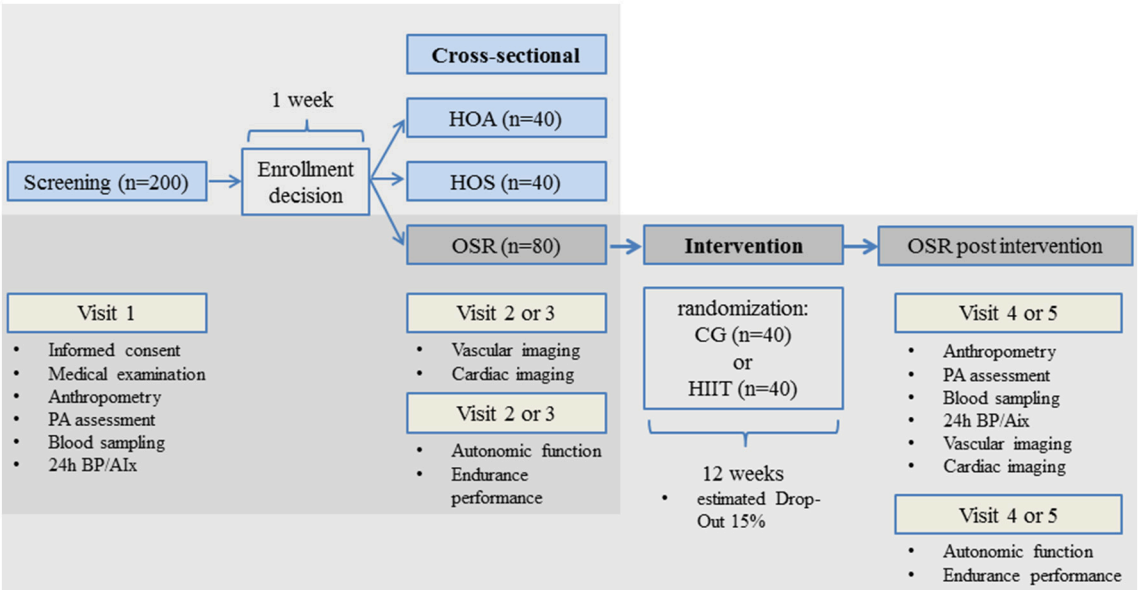

FIGURE 1 | Study design, cross-sectional part (Part I) and randomized controlled trail (RCT) (Part II); HOA, healthy older active; HOS, healthy older sedentary; OSR, older sedentary at risk; CG, control group (standard recommendations for daily physical activity); HIIT, high intensity interval training.

All participants are committed to sign a consent form and will be informed about their right to withdraw from the study without any consequences. A study assistant will randomize the OSR group in an intervention or a control group by drawing a lot after the last pre-intervention assessment. Participants and investigators are blinded for the group membership during the pre-measurements. All participants are to be recruited from our outpatient department, the Metabolism Unit of the University Hospital Basel, Basel, Switzerland and, in addition, with postings on University webpages and advertisements in local newspapers. We aim to recruit the HOA group in running clubs and on running events in and around Basel. A total of 160 participants will be recruited for the study.

Statistical Analysis

The primary outcome of the study is the cfPWV among HOA, HOS, and OSR (part I), and among OSR after 12 weeks of intervention with a high-intensity interval training (HIIT) and those assessed within the same time frame without the HIIT intervention (control group) (part II). To describe continuous demographic and baseline characteristics of participants in the HOA, HOS and OSR group (part I) and those in the OSR intervention and control group (part II), we will use the median and interquartile range; and for categorical characteristics, we will use percentages. Boxplots will be used to visualize the primary and secondary outcomes in the HOA, HOS and OSR group (part I) and at baseline as well as after 12 weeks in the OSR intervention and control group (part II). For part I, we will use analysis of variance to compare the cfPWV (and secondary outcomes) between HOA, HOS and OSR. For part II, we will use analysis of covariance to compare the cfPWV (and secondary outcomes) after 12 weeks between OSR in the intervention and those in the control group adjusted for the corresponding values at baseline (Vickers and Altman, 2001). For each analysis, we will report estimates (with 95% confidence intervals) of the difference in outcome between HOA and HOS and between OSR and HOS (part I), and between OSR in the intervention and those in the control group (part II). Up-to-date versions of SAS (SAS Institute Inc., Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria) will be used for analysis and graphics.

Sample Size Calculation

The sample size was calculated separately for both study parts. For part I, we assumed that the expected cfPWV corresponded to 8.5, 9.5, and 11.5 m/s for HOA, HOS and OSR, respectively, and that the standard deviation was 1.5 m/s (Tedesco et al., 2004; Gando et al., 2010). With a 2-sided significance level of 0.05, the sample size needed to attain a targeted power of 80% for detecting a difference with magnitude 1.0 m/s was 36 participants per group (the comparison between HOA and HOS) and for detecting a difference with magnitude 2.0 m/s, it was 10 participants per group (the comparison between OSR and HOS). For part II, we assumed that the expected difference in cfPWV after 12 weeks between OSR in the intervention and those in the control group was 1.0 m/s and that the standard deviation was 1.5 m/s (Madden et al., 2009). By including the baseline cfPWV (before the start of the intervention period) as a covariate in the pre-specified analysis, we will further reduce error variability and therefore assumed that the correlation between baseline and outcome cfPWV was 0.3. With a 2-sided significance level of 0.05, the sample needed to attain a

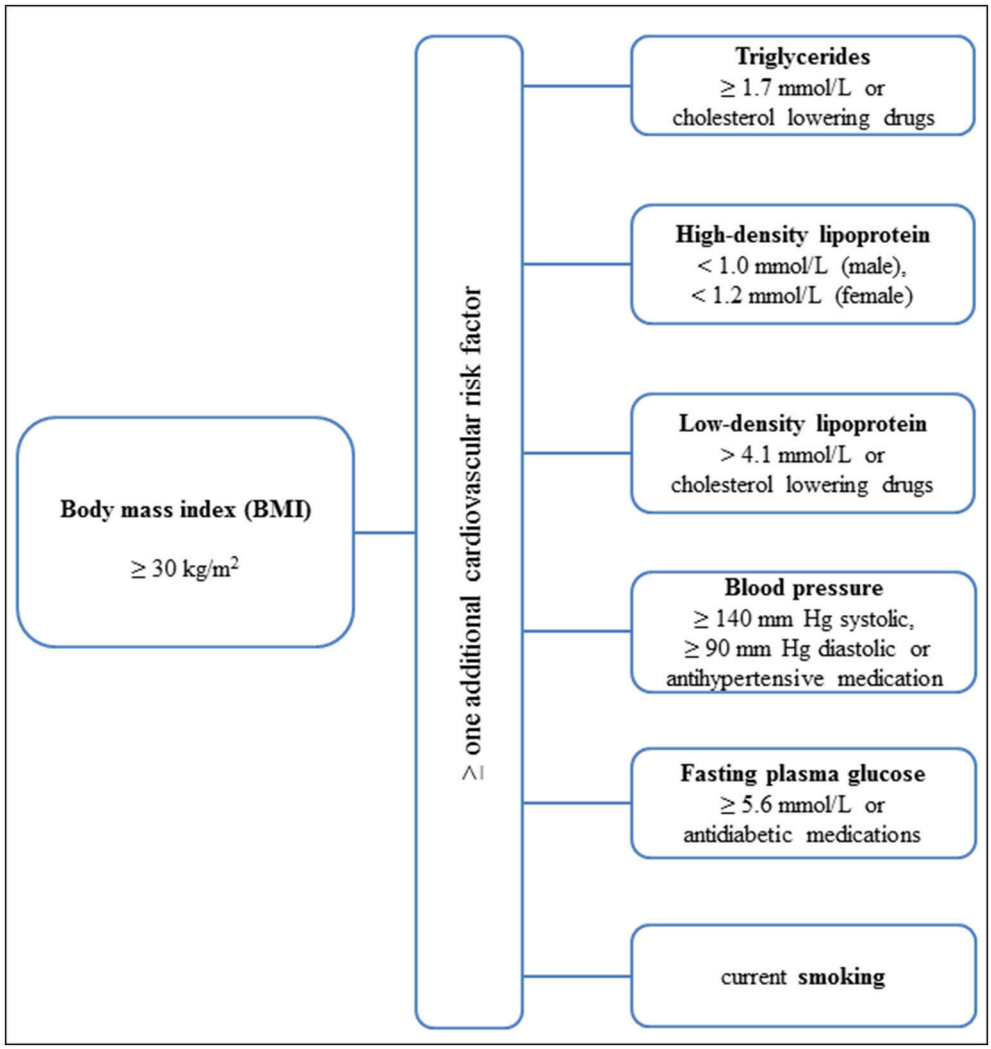

FIGURE 2 | Inclusion criteria and risk factors for older sedentary at risk group (OSR).

targeted power of 80% for showing superiority of the intervention over control was 34 participants per group. Taking dropouts into account, we plan to include a total of 40 HOA, 40 HOS and 80 OSR. The POWER and GLMPOWER procedures in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) were used for sample size calculation for the first and second study part, respectively.

Exercise Intervention and Control Condition

High Intensive Interval Training (HIIT)

The exercise intervention for the OSR intervention group is a supervised Nordic-Walking training three times a week with gradually increased intensity during the first 2 weeks. In the first week, we will teach Nordic Walking technique, warm-up and cool-down elements as well as continuous walking training with moderate intensity at 75% of maximal heart rate (HRmax). In the second week, the intensity will increase to 80–90% of HRmax. In the following 10 weeks, HIIT will be performed as described below. Exercise scientists will supervise all training sessions.

The following protocol will be used (modified from Wisloff et al., 2007):

- Three supervised trainings per week
- Intensity: 10 min warm-up at 65-70% HRmax, 4x4 min interval training at 80-90% HRmax with 3 min of active recovery at 65-70% HRmax, 10 min cool down at 60-70% HRmax
- Duration: 45 min

Control Group (CG)

The control group is asked to orientate their physical activity according to the European Guidelines on vascular disease prevention in clinical practice (Backer et al., 2003).

IMAGING METHODS AND COURSE OF MEASUREMENTS

Macrovascular Aging

Central Pulse Wave Velocity

Carotid-femoral PWV (cfPWV), the gold standard for the measurement of arterial stiffness, is assessed by using a SphygmoCor® device (AtCor, Medical Pty Ltd, Sydney, Australia). The good intra- and interobserver reproducibility of this technique has been demonstrated in healthy populations and in patients with chronic kidney disease (Frimodt-Møller et al., 2006, 2008). After 10 min of rest, pulse waves are recorded using a high-fidelity tonometric transducer at two sites (right carotid artery and right femoral artery). Pulse wave travel distance is determined by subtracting the distance between the manubrium to the carotid artery from the distance between the femoral artery to the manubrium as previously described (Townsend et al., 2015). cfPWV measurements are considered to meet the quality control parameters if two consecutive measurements are visually acceptable and within 1 m/s of each other with a standard deviation of <10% (Sigrist et al., 2010). If this criterium could not be reached a third measurement was applied. The mean of all valid measurements represents the cfPWV value.

Peripheral Pulse Wave Velocity

Femoral-tibial PWV (ftPWV) is assessed by using the same SphygmoCor® device as for central PWV. Peripheral PWV is determined as the travel time of the pulse wave between the two recording sites at the femoral artery and the posterior tibial artery divided by the distance between the two recording sites. All other procedures and conditions are the same as for central PWV (see above).

Pulse Wave Reflection (PWA)

AIx and AIx@75 are measured using the gold standard device for single measurements (SphygmoCor®). The augmentation index from the central pulse is calculated as: AIx = 100 × (P2 – P1)/pulse pressure, where P2 is the peak of the reflected backward wave, P1 is the peak of the forward pressure wave and central pulse pressure is the systolic pressure maximum minus the diastolic pressure. A positive AIx would indicate an augmentation of peak systolic pressure by the reflected wave. Pulse waveform is obtained using applanation tonometry (SphygmoCor® device, ATCor Medical, Sydney, Australia) on the right radial artery. Ten-second recordings are accumulated until either two analyses with a quality index of ≥90% or three of ≥80% were available. By applying a generalized transfer functions (GTF) the central arterial pulse waveform is estimated by the SphygmoCor® software. These transfer functions have been validated previously during rest (Gallagher et al., 2004) and exercise (Sharman et al., 2006). The calculated central arterial pulse waveform is subjected to further analysis, such as the determination of AIx and AIx@75. Reproducibility has been shown to be acceptable at rest (Filipovský et al., 2000) and during exercise (Holland et al., 2008).

24-h Ambulatory Monitoring of Central Hemodynamics

Twenty-four hours ambulatory pulse wave monitoring is obtained using an oscillometric Mobil-O-Graph® 24 h PWA Monitor device (I.E.M GmbH, Germany) with integrated ARCSolver® software. Based on the oscillometric data, central hemodynamics as well as the 24-h PWV is calculated. Oscillometric pulse wave analysis are performed every 20 min for 24 h. Subjects are instructed to hold their arm as steady as possible during the measurements but otherwise maintain their daily routine with no additional physical activity while wearing the device. After data readout, every individual measurement is reviewed for erroneous values. Values are deleted if the quality of data is graded 3 or 4 by the ARCSolver® software. The methods used for these analyses are the same as used by the SphygmoCor® software described previously (Wassertheurer et al., 2010). Validity and reproducibility of oscillometric estimates of cPP, Aix, and AIx@75 are comparable with laboratory settings (Wassertheurer et al., 2010; Luzardo et al., 2012; Papaioannou et al., 2013).

Intima Media Thickness (IMT)

For the semi-automatic evaluation of intima-media thickness (IMT), B-Mode clips are conducted using an ultrasonic device UF-870AG (Fukuda Denshi, Japan) according to current guidelines (Touboul et al., 2012). With the participant in supine position and the head rotated by 45° either to the left or right side two locations on both sides are scanned. Ear-to-ear and horizontal clips are recorded over at least three heart cycles using the US machines inbuilt 3-lead ecg function. Post procession and video-based IMT-admeasurement of the exported clips is performed using the Dynamic Artery Analysis software (DYARA) as described elsewhere (Teynor et al., 2012), using image averaging over three heart cycles. Reproducibility of the aforementioned method is excellent (Touboul et al., 2012).

Microvascular Aging

Static Retinal Vessel Analysis (SVA)

The retinal microcirculation is easily accessible by using the Retinal Vessel Analyser (RVA, IMEDOS Systems, Jena, Germany) and a fundus camera (450 FF; Carl Zeiss, Jena, Germany). The technique allows for the analysis of the structure and function of retinal arterioles and venules. To measure retinal vessel diameters, we analyze three valid pictures from one eye with an angle of 50° and the optic disc in the center. The detailed procedure is described elsewhere (Hanssen et al., 2011). Briefly, retinal arterioles and venules, coursing through an area of 0.5–1 disc-diameter from the optic disc margin, will be identified semi-automatically at higher magnification using special analyzing software (Vesselmap 2, Visualis, Imedos Systems UG). Diameters will be averaged to central retinal arteriolar (CRAE) and venular (CRVE) equivalents, and the arteriolar-to-venular-ratio (AVR) will be calculated from the CRAE and CRVE. The inter-observer and intra-observer interclass correlation coefficient for the measurement of retinal vessel diameters ranges from 0.75 to 0.99 (Hubbard et al., 1999; Wong et al., 2004b).

Dynamic Retinal Vessel Analysis (DVA)

Functional retinal vessel analysis requires pharmacological dilatation of one pupil with conventional eye drops (Tropicamide 0.5%). Microvascular function is analyzed by a flicker-induced dilatation of retinal arterioles and veins. We use a fundus camera (450 FF; Carl Zeiss, Jena, Germany), with a charge-coupled device for electronic online imaging and a personal computer for system control, analysis and recording of the obtained data, allowing non-invasive assessment of retinal endothelial function mediated by neurovascular coupling following flicker stimulation (Falsini et al., 2002; Polak et al., 2002). The whole procedure is described elsewhere (Nagel et al., 2004; Gugleta et al., 2006; Garhofer et al., 2010). Due to the described inter- and intra-individual variations of vessel diameters (Hubbard et al., 1999), the mean diameter resulting from three baseline measurements before application of the stimulus in each of three flicker cycles is defined as 100%. The ensuing vessel diameter changes are recalculated in % to this individual baseline value (Pemp et al., 2009). From the median of the three curves, parameters such as maximal vessel dilatation, maximal reactive vessel constriction and area under the reaction curve during and after flicker stimulation can be analyzed (Kotliar et al., 2011).

Cardiac Aging

Echocardiographic parameters are assessed using an ultrasonic device (UF 870AG, Fukuda Denshi, Japan) according to current guidelines (Nagueh et al., 2009; Lang et al., 2015). Briefly, global systolic function is described by ejection fraction based on chamber quantifications as recommended. Linear method and 2D based formulas are used to calculate left ventricular function mass. Right ventricular measurements (such as tricuspid annular plane systolic excursion) as well as atrial volume measurements give further insight into mechanical properties of the heart. Diastolic function is assessed using mitral inflow patterns (E, A, deceleration time, intraventricular relaxation time), tissue Doppler derived mitral annular velocities (E′, A′) and pulmonary artery pressures. Additionally, speckle tracking echocardiography is used to calculate strain.

Autonomic Aging

We record a 12-lead-ECG for 20 min in supine position before and 10 min after a treadmill test using Custo Diagnostic Software (Custo Med GmbH, Germany). The additional post-treadmill assessment will enable us to evaluate recovery of HRV after acute bouts of exhaustive exercise. Raw data are extracted and processed to derive HRV parameters. Time-domain parameters are standard deviation of normal to normal (NN) RR intervals (SDNN), root mean square of successive differences (RMSSD) and the number of pairs of successive NNs that differ by more than 50 ms (NN50). Frequency-domain paramters include high- and low frequency power. Additionally, Heart Rate Turbulence (HRT) and Deceleration Capacity (DC) are calculated (Schmidt et al., 1999; Bauer et al., 2006).

Inflamm-Aging and Circulating CV Risk Factors

To analyse the influence of physical activity on immunological processes and the association of inflammation, blood samples are taken. They are drawn by venepuncture of the cubital fossa of the right or left arm by trained medical staff in a fasting state. The blood is transported directly to the clinical chemistry laboratory for further analysis or centrifuged and put on ice. Clinical routine measurements including total blood count, total cholesterol (TC), low- (LDL) and high-density lipoprotein (HDL), triglyzerides (TGA) (colorimetric tests) as well as fasting glucose levels (hexokinase reference method) and insulin levels (automated immunoanalyzer system) to estimate insulin resistance (by HOMA Score) are measured. Samples, which are not analyzed directly, will centrifuged and the plasma aliquots will be frozen at a temperature of −80°. To analyse plasma concentration of the following specific biomarkers blood samples will be defrosted and processed. Cytokine-specific ELISA kits will use to analyse inflammatory serum biomarker interleukin 6 (IL 6), 10 (IL-10), and tumor necrosis factor alpha (TNF-α) according to the manufactures instructions (IL-6/-10: Bender Med-Systems (eBioscience), Austria; TNF-α: Bio-source, USA). The samples will be distributed in duplicates on the plates and the intra-assay and inter-assay coefficient of variation need to be $\leq$10%. High-sensitive c-reactive protein (hsCRP) will be analyzed by immunoturbidimetric latex CRP assay (Cobas 8000, Roche-Diagnostics, Basel).

Physical Fitness, Physical Activity, and Anthropometry

Physical fitness will be assessed by performing an individualized ramp protocol on the treadmill. We will measure peak oxygen uptake (VO2peak) and maximal heart rate (HRmax). The individualized design with increasing speed and ramp steepness will be used to reach volitional exhaustion after approximately 10 min for every participant. The calculation of this protocol is based on the subject's age and estimated peak metabolic equivalent units (METS) as previously recommended (Bader et al., 1999; Myers and Bellin, 2000). The ECG will be monitored by medical personal during the whole test. Blood pressure will be measured during, immediately after and 3 min after the exercise test. Every minute Ratings of Perceived Exertion (RPE) will be requested (Borg, 1982). Ventilatory parameters, including VO2max and heart rate, will be measured by using the Cortex Metalyzer® 3B metabolic test system (Cortex Biophysik GmbH, Leipzig, Germany).

All participants will get an accelerometer (Aipermon GmbH, Germany) to analyse their physical activity (PA). They have to wear the device on their left hip for the whole day on six consecutive days. Raw data will be copied onto a computer and the data will be viewed using the ActiCoach MPAT2Viewer (Aipermon, GmbH, Germany). The following parameters can be analyzed by this software: total time (minutes per day) spent passively (PAS), actively (ACT), walking (WLK), and fast walking (FWLK). Walking and fast walking times will be joined to a total walking time (TWT). We have previously validated activity modes and accelerometer detection precision (Jehn et al., 2009a,b, 2010, 2011).

Self-reported PA will be measured with the Freiburg Questionnaire of physical activity. The questionnaire interrogates health-related physical activities using self-reported activities

within the last week/month. It creates reliable and valid values of PA and inactivity (Frey et al., 1999). The total PA is expressed in hours per week. The Ainsworth Compendium in metabolic equivalents (METS) estimates the intensities. The formula to calculate the energy expenditure per week is described elsewhere (Ainsworth et al., 1993).

Anthropometric data will be measured in a fasting state in the morning. The Inbody 720® (JP Global Markets GmbH, Germany) device will be used to obtain weight, BMI, skeletal muscle mass and body fat (Anderson et al., 2012). Height and waist circumference will be measured by using a normal measuring tape with respect to current guidelines.

Potential Pitfalls

Our main outcome (cfPWV) as well as most other vascular measurements are affected by age, hemodynamic variability, lifestyle behavior, and circadian fluctuations. To minimize external interference and variability, several measures are considered. Groups are matched for age and sex. Standardized vascular imaging is performed under fasting conditions in the morning after 10 min of rest in a supine position. Participants are encouraged to refrain from alcohol, caffeine and if applicable from smoking 12 h prior to the examinations. Exercise and vigorous physical activity should not be performed 24 h before the visits. The OSR group is allowed to take their medication in the morning. These measures help to minimize intra-individual as well as within-day variability.

Change of medication or start of a structured diet during the 12 week-week intervention or control condition are indications for exclusion. In addition, the start of structured exercise in the control group is considered an exclusion criterion. To prevent the start of exercise in the control group, all participants of this group are invited to take part in the voluntary supervised exercise program after the post measurements. To prevent high drop-out rates and optimize adherence to the exercise regime, every exercise session starts with strength, mobilization and coordination exercises and includes a warm-up and a cool-down. The first 2 weeks of the exercise program focus on technical and coordinative skills at lower intensities before HIIT is started in the third week into the program.

DISCUSSION AND CONCLUSION

The study design offers a concept for a systems physiology approach on the mechanisms of aging and the role of physical activity and fitness. The cross-sectional approach enables to investigate the association of physical activity with the process of normal, healthy aging comparing the HOA group with HOS. In addition, the impact of cardiovascular disease on physiological functioning is examined by comparing the HOS group with OSR. The reversibility of pathophysiological aging in patients with cardiovascular risk is explored by applying an interventional exercise treatment in the OSR group. Our study concept investigates the interplay of physiological systems and how these are affected by physical activity and fitness. To what extent can physical fitness prevent or delay physiological dysfunction? The study aims at clarifying some of the main mechanisms by which exercise improves the process of aging. The multimodal concept of the study is depicted in **Figure 3**.

The ExAMIN AGE Study resembles new approaches in the structural and functional diagnostics of aging processes including systems such as the vasculature, the heart, autonomic function and inflammation. The results have a focus on vascular imaging and on the non-pharmaceutical treatment of vascular health in older inactive individuals at risk. The integrative approach will help to determine the best non-invasive means to diagnose

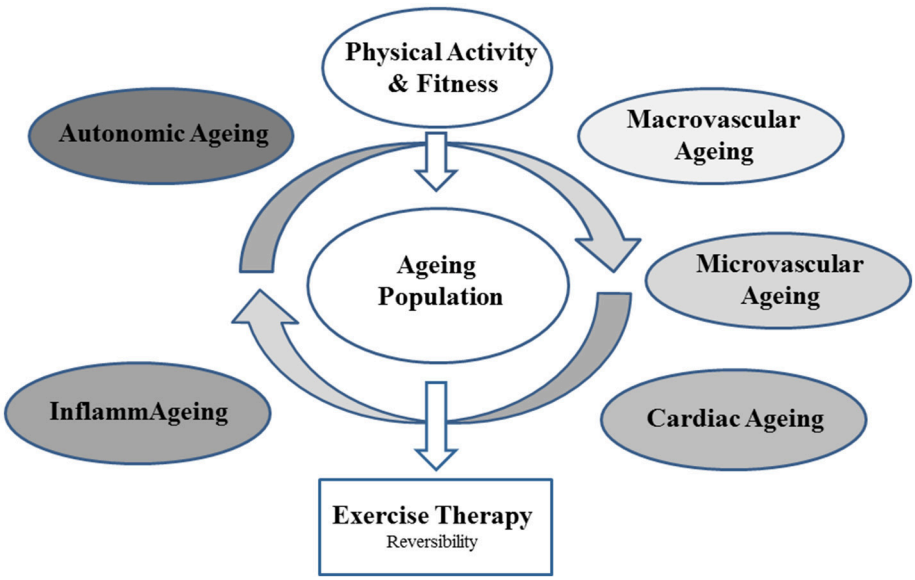

FIGURE 3 | Principle concept of the systems physiology approach.

and define cardiovascular risk in advanced age and disease. This study design allows the examination of pathophysiological links between small and large arteries, the so called "vascular coupling" or "arterial cross-talk." To date little is known about the interaction between the two vascular beds and how they affect each other. It also enables to examine the cross-talk between the vasculature and the heart. Exercise interventions can improve both entities and play a key role in the treatment of cardiovascular and metabolic diseases.

Our study has some limitations. We are aware that the recruitment of healthy older sedentary individuals (HOS) is challenging. It needs to be ascertained that all three groups are strictly matched for age. The analysis of the association of physical fitness with cardiovascular health and aging processes in the three groups represents a cross-sectional approach and is not based on prospective longitudinal data. Life-long activity is analyzed by subjective reporting but is supported by objective analysis using accelerometry and spiroergometry. The cross-sectional study design does not give evidence of a temporal relationship between physical activity exposure and physiological outcome. However, the interventional part of the study, where participants are randomly allocated to the intervention or the control group, allows for a causal and temporal analysis of the improvement of aging processes in individuals at risk. The extensive phenotyping of vascular health and other physiological systems as well as the analysis of the impact of HIIT are the main strengths and innovations of the study.

In conclusion, the concept links cardiovascular prevention and exercise medicine in a systems physiology approach. It aims to help define new recommendations for treatment guidance of exercise therapy in an aging population. We aim to demonstrate the importance of specific physical activity programs for seniors to achieve healthier aging as a long-term goal. Amelioration of vascular function represents improvement of disease- and age-related end organ damage and best describes the potential to contain vascular health. The study will generate results which will help to understand better the mechanisms of vascular aging and the interplay with other physiological systems. The study approach has the potential for transfer in other age groups and clinical settings.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Helsinki Declaration and the SPIRIT Guidelines. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351).

AUTHOR CONTRIBUTIONS

LS: drafted the manuscript, performed examinations, is responsible for general data collection and analysis of retinal vessels and cardio respiratory fitness, organized and performed the exercise interventions; AD: helped draft the manuscript, performed the large artery measurements as well as cardiac imaging, medical examination, and supervision of all participants; JS: performed the sample size estimation and was responsible for statistical considerations; AS-T: participated in the study design and helped draft the manuscript; HH: designed the study and is principal investigator, wrote the manuscript and helped analyse the data. All authors read and approved the final manuscript.

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The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3. Publication 2: High-intensity interval training modulates retinal microvascular phenotype and DNA methylation of $p66^{Shc}$ gene: a randomized controlled trial (EXAMIN AGE)

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Abstract

Aims:

Impairments of retinal vessel diameter are associated with major adverse cardiovascular (CV) events. Promoter DNA methylation is a repressor of the mitochondrial adaptor $p66^{Shc}$ gene transcription, a key driver of ageing-induced reactive oxygen species. The study aimed to investigate whether high-intensity interval training (HIIT) affects retinal microvascular phenotype as well as $p66^{Shc}$ expression and oxidative stress in ageing subjects with increased CV risk from the EXAMIN AGE cohort.

Methods and results:

Eighty-four sedentary subjects (mean age 59.4±7.0 years) with ≥ two CV risk factors were randomized into either a 12-week HIIT or standard physical activity recommendations. Retinal arteriolar and venular diameters were measured by use of a retinal vessel analyser. As a marker of oxidative stress plasma 3-nitrotyrosine (3-NT) level was determined by ELISA. Gene expression of $p66^{Shc}$ and DNA methylation were assessed in mononuclear cells by RT-qPCR and methylated-DNA capture (MethylMiner Enrichment Kit) coupled with qPCR, respectively. HIIT reduced body mass index, fat mass, low-density lipoprotein and increased muscle mass as well as maximal oxygen uptake (VO2max). Moreover, HIIT restored microvascular phenotype by inducing retinal arteriolar widening (pre: 175±14µm vs post: 181±13µm, p=0.001) and venular narrowing (pre: 222±14µm vs post: 220±14µm, p=0.007). After HIIT, restoration of $p66^{Shc}$ promoter methylation (p=0.034) reduced $p66^{Shc}$ gene expression (p=0.037) and, in turn, blunted 3-NT plasma levels (p=0.002).

Conclusion:

HIIT rescues microvascular dysfunction in ageing subjects at increased CV risk. Exercise-induced reprogramming of DNA methylation of $p66^{Shc}$ gene may represent a putative mechanistic link whereby exercise protects against age-related oxidative stress.

Introduction

Retinal vessels are part of the cerebrovascular bed and represent an accessible window to investigate microvascular health and subclinical vascular remodelling. The retinal microcirculation has previously been described as a window to the heart[1]. Alterations of the retinal microvascular phenotype are associated with heart failure[2] and coronary heart disease mortality[3] and have been shown to be predictive of long-term CV outcome in the general population[4]. In ageing subjects, narrower retinal arterioles and wider venules have been associated with increased CV events such as stroke[5], coronary artery disease[6] and higher CV mortality[7]. Physical inactivity has been associated with worse retinal microvascular phenotype[8].

A key feature of vascular ageing is the imbalance between NO bioavailability and reactive oxygen species (ROS) leading to endothelial dysfunction, early step in the pathogenesis of CV events. In this setting, the adaptor protein $p66^{Shc}$ has emerged as an important redox enzyme implicated in mitochondrial ROS generation. In patients with type 2 diabetes (T2DM), we previously found that upregulation of $p66^{Shc}$ correlates with urinary 8-iso-prostaglandin F2 levels, an *in vivo* marker of oxidative stress, and endothelial dysfunction[9]. Epigenetic regulation of gene transcription is mediated primarily by DNA methylation and posttranslational modifications of histone proteins[10]. While the benefits of physical activity are widely acknowledged, the role of exercise-induced epigenetic regulation of genes implicated in vascular ageing remains poorly understood. Regular exercise can modulate methylation levels which translate into differential gene expression at genome-wide level in healthy men and women[11]. High-intensity interval training (HIIT) can improve endothelial function and cardiorespiratory fitness comparable or even superior to moderate continuous training[12]. However, the impact of exercise on the interplay between microvascular phenotype, ROS generation and $p66^{Shc}$ gene transcription is unknown. This randomized controlled trial was designed to assess the effects of HIIT on retinal vessel diameters as a microvascular biomarker of CV risk as well as DNA methylation of $p66^{Shc}$ gene and oxidative stress in ageing subjects with CV risk from the EXAMIN AGE cohort[13].

Material and Methods

The complete methods section of this randomized controlled trial is available in the supplementary text online including the power calculation and sample size estimation.

Results

We recruited 452 participants through our Outpatient Prevention Clinic and advertisements in local newspapers in and around the City of Basel. Eighty-four patients (mean age 59 ± 7 years, 42 female) met the inclusion criteria and were randomized into HIIT or standard physical activity recommendations (control group). Forty subjects undergoing HIIT and 34 controls were measured post-intervention (Supplementary Material online, Figure S1). No changes in medication during the HIIT-period or HIIT-related adverse events were observed. Distribution of CV risk factors is listed in Supplementary Material online, Figure S2. Anthropometric measurements before and after 12 weeks of intervention or standard physical activity are listed in Supplementary Material online, Table S1.

Microvascular phenotype

After 12 weeks of HIIT, retinal arteriolar diameters significantly increased (pre: 175 ±14µm *vs* post: 181 ±13µm, p=0.001) and venular diameters decreased (pre: 222 ±14µm *vs* post: 220 ±14µm, p=0.007) as compared to the control group (Figure 1A, Supplementary Material online Table S2). Further adjustment for maximal oxygen uptake (VO2max) showed that arteriolar widening and venular narrowing were dependent on changes in VO2max. The increased arteriolar-to-venular diameter ratio (AVR) in the intervention group (β (95% confidence interval): 0.03 (0.01; 0.05), p=0.005) was independent of age, change (Δ) in body mass index (BMI), systolic and diastolic blood pressure, CV medication and ΔVO2max (Supplementary Material online, Table S2). Retinal diameters did not change in the control group. We also performed an intention-to-treat analysis for the primary outcome. The exercise-induced effects on retinal arteriolar diameter and arteriolar-to-venular diameter ratio (AVR) remained significant but not the effect of venular narrowing (Supplementary Material online, Table S3).

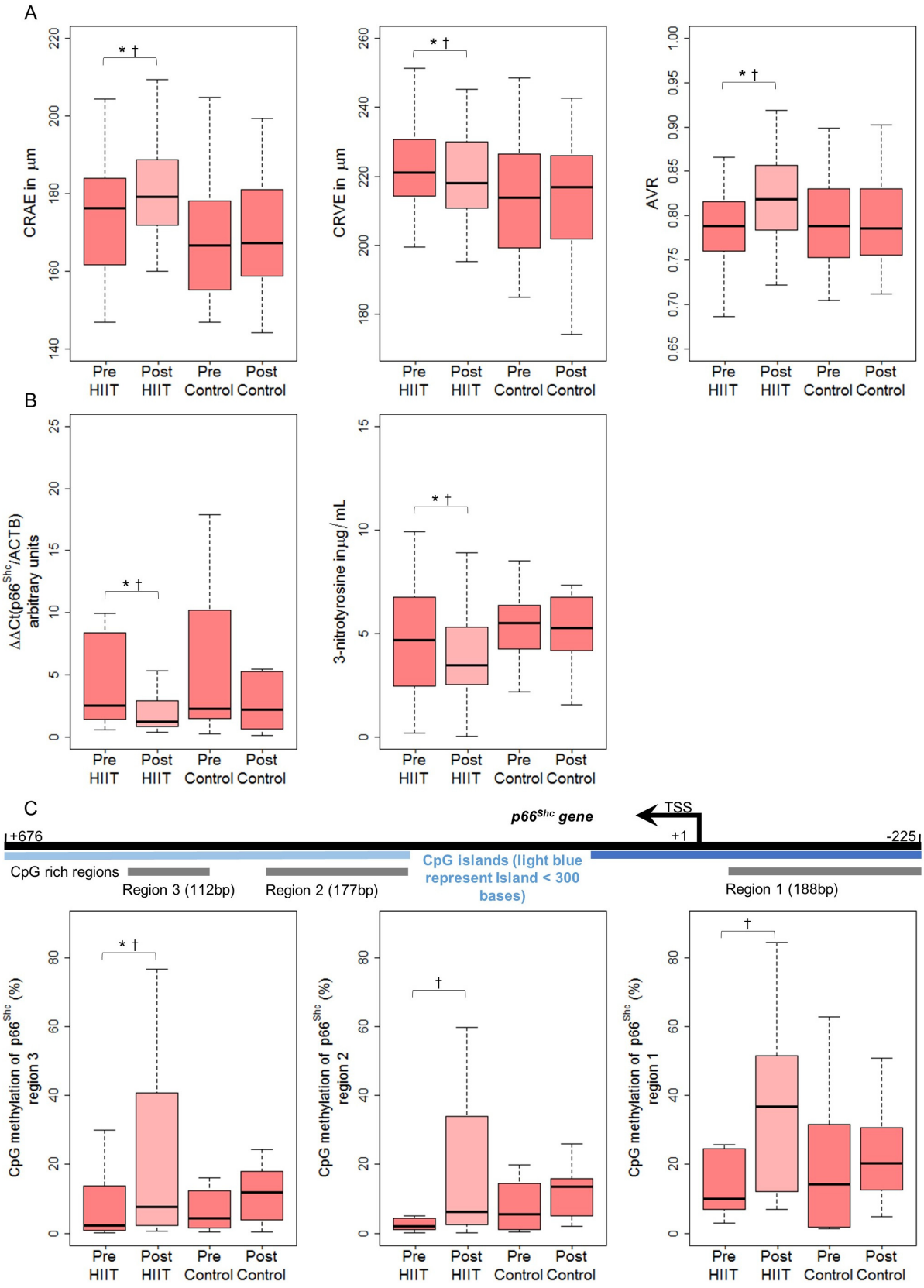

Figure 1.

(A) Retinal vessel diameters in subjects with increased CV risk before and after intervention. 40 subjects before and after HIIT and 34 before and after standard physical activity

recommendations (control group) were included in the final analysis. (B) Expression of $p66^{Shc}$ relative to ACTB was measured before and after HIIT or standard physical activity recommendations in 20 subjects randomly selected from each group. Oxidative stress measured as plasma 3-NT levels in 40 subjects before and after HIIT and 34 before and after standard physical activity recommendations. (C) $p66^{Shc}$ gene and CpG islands proximal to $p66^{Shc}$ promoter (grey lines indicate CpG rich regions amplified with specific primers). Levels of DNA methylation at region 3, 2 and 1 of $p66^{Shc}$ promoter were measured in the same 20 subjects before and after HIIT or standard physical activity recommendations. Values are expressed as mean±SD. ANCOVA p-values corrected for baseline and control group are shown for multiple comparisons. *$p<0.05$ for ANCOVA and between group differences; $^{\dagger}p<0.05$ for t-test and within group differences. CV, cardiovascular; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar to venular diameter ratio; HIIT, high intensity interval training; Ct, cycle threshold; ACTB, actin beta.

Oxidative stress and $p66^{Shc}$ expression

12 weeks of HIIT as compared to control condition significantly reduced mitochondrial adaptor $p66^{Shc}$ gene expression in peripheral blood mononuclear cells (pre: 6.5 ±8.4 arbitrary units (AU) *vs* post: 1.9 ±1.5 AU, p=0.037) and blunted 3-nitrotyrosine (3-NT) plasma levels (pre: 5.6 ±5.1 µg/ml *vs* post: 3.8 ±2.2 µg/ml, p=0.002) after adjustment for age, Δ BMI, systolic and diastolic blood pressure, CV medication and ΔVO2max (Figure 1B, Supplementary Material online, Table S2).

DNA methylation of $p66^{Shc}$ gene

$p66^{Shc}$ promoter was analyzed for DNA methylation using Methylminer and qPCR. Three different sets of primers were designed to amplify the CPG islands in the $p66^{Shc}$ promoter (-225/+676 bp of the transcription start site (TSS)) and to comprehensively examine the methylation status of the CpG islands in the two experimental groups (Figure 1C). A significant restoration of DNA methylation status of $p66^{Shc}$ promoter was observed in all three regions in the HIIT group ($p<0.05$, within group analysis). By contrast, no changes were found in the control group. Following adjustment for confounders DNA methylation levels in region 3 remained significantly upregulated after HIIT (p=0.034, Figure 1C).

Discussion

Twelve weeks of HIIT improved retinal microvascular phenotype. Moreover, HIIT-induced reprogramming of DNA methylation of $p66^{Shc}$ gene was associated with downregulation of $p66^{Shc}$ expression in peripheral blood mononuclear cells and subsequent decrease of systemic oxidative stress. Improvements in retinal AVR, $p66^{Shc}$ gene expression and oxidative stress were independent of age, ΔBMI, systolic and diastolic blood pressure, CV medication and ΔVO2max.

Our randomized controlled trial demonstrates that microvascular remodelling in an older population with increased CV risk can be reversed by short-term exercise training. HIIT resulted in arteriolar widening and venular narrowing. In the intention-to-treat analysis, exercise-induced arteriolar widening but not venular narrowing remained significant suggesting predominant effects on arterioles. This retinal microvascular phenotype has been linked with a lower incidence of CV events and CV mortality[4, 14, 7]. Reduced oxidative stress with subsequent restoration of NO bioavailability are major drivers of exercise-induced improvement of microvascular function. Indeed, endurance exercise has the capacity to directly increase endothelial NO production. We have previously shown that exercise-induced dilatation of retinal arteriolar diameters in obese individuals was accompanied by a reduction of asymmetrical dimethyl-L-arginine (ADMA), an endogenous inhibitor of the L-arginine/NO pathway[15]. HIIT has been reported to improve antioxidant capacity and vascular reactivity more potently than moderate continuous training[12]. Higher blood flow and shear stress during HIIT increase plasma glutathione peroxidase availability, which may contribute to ROS reduction and, in turn, increased NO bioavailability[16]. In patients with heart failure, exercise-induced attenuation of oxidative stress has been associated with improvement of vascular function[12]. Endothelial homeostasis depends in large part on the balance between oxidant and antioxidant pathways. Higher shear stress during HIIT has been shown to result in more distinct improvement of endothelial function[12]. Indeed, post-exercise AVR was restored independent of potential confounders and improvement of microvascular endothelial function may be a key mechanism involved.

Direct effects of exercise on clinical risk factors may also impact microvascular health. Blood pressure and fasting glucose levels did not change significantly during exercise training. In patients with diabetes, hyperglycaemia is associated with increased $p66^{Shc}$ gene expression[9].

In our study, p66^{Shc} expression was blunted after exercise without any modification in fasting blood glucose, indicating that exercise-induced changes in p66^{Shc} gene expression are not necessarily linked to blood glucose levels. The observed changes in body composition with a decrease in fat mass as well as the reduction of LDL-cholesterol levels may contribute to the observed improvement of microvascular function. Interestingly, we adjusted our analyses for BMI, CV medications including other potential confounders and the results remained significant. In view of the different beneficial effects elicited by HIIT, the underlying mechanisms responsible for the restoration of microvascular phenotype are likely to be multifactorial. Future studies are warranted to investigate the relative contribution of direct and indirect exercise-induced effects on the vasculature.

This is the first study in ageing subjects with increased CV risk to report that exercise-induced downregulation of p66^{Shc} gene expression is associated with a reduction of oxidative stress. In this regard, it is well established that $ONOO^-$ formation originating from the reaction of NO and O_2^- contributes to increased 3-NT plasma levels[17, 18]. This study shows that HIIT, by rescuing methylation of p66^{Shc} promoter, can reduce p66^{Shc} gene transcription that, in turn, may contribute to a decrease of 3-NT plasma levels. We have previously demonstrated in experimental models of diabetes and in patients with T2DM that hypomethylation of p66^{Shc} promoter causes gene overexpression, oxidative stress and endothelial dysfunction[19, 7]. In addition, genetic deletion of p66^{Shc} protects against age-induced, ROS-mediated endothelial dysfunction, most likely by restoring NO bioavailability[20].

Our intervention trial investigates for the first time the interconnection between exercise, microvascular phenotype and epigenetics in ageing subjects with increased CV risk. Interestingly, p66^{Shc} methylation is dynamic showing robust changes in the post-HIIT group. Our study provides a proof of concept that epigenetic regulation of p66^{Shc} is related to age-induced oxidative stress and microvascular phenotype. Although only part of the epigenetic crosstalk has been elucidated, the present findings shed light on a key epigenetic mark linking exercise-induced reprogramming of p66^{Shc} expression, decreased ROS generation and improved microvascular health. The conclusion of our work is summarized in Figure 2. Previous evidence suggest mechanistic links between p66^{Shc} transcription, oxidative stress and endothelial function[9, 20, 21]. It is therefore plausible to hypothesize that exercise-induced downregulation of p66^{Shc} transcription via DNA methylation contributes to rescue

microvascular function. Future research has to prove the causative link in this setting. The disentanglement of single molecular pathways with their systemic impact and potential effects on complex organ function in humans remains a major scientific challenge.

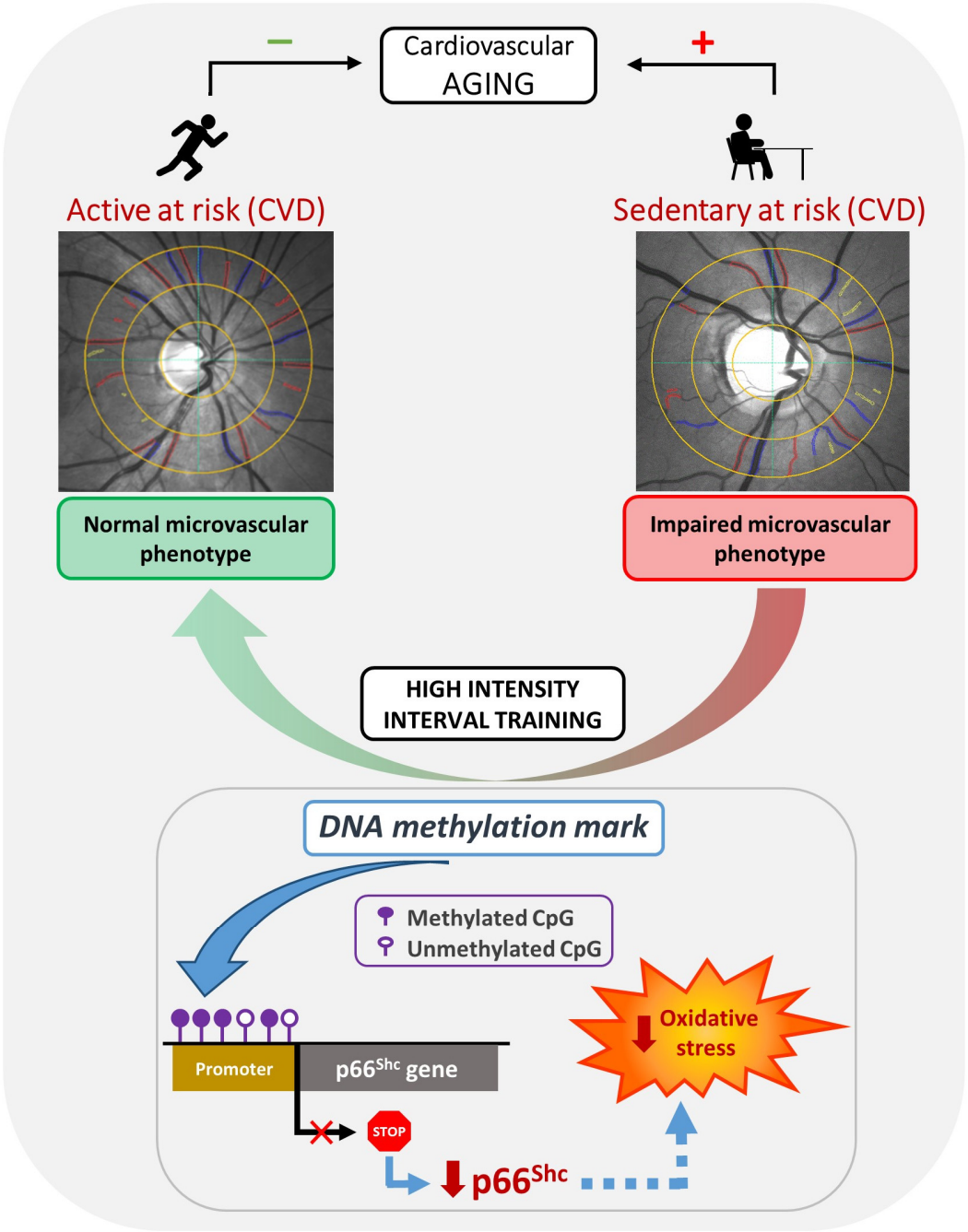

Figure 2.

Take-home figure shows exercise-induced improvement of microvascular phenotype and reprogramming of $p66^{Shc}$ DNA methylation. HIIT improves microvascular phenotype. Reprogramming of DNA methylation mark on $p66^{Shc}$ gene promoter may represent a

mechanistic link whereby physical exercise protects against age-related oxidative stress in the microcirculation. HIIT, high intensity interval training.

Our study validates a new multidisciplinary research perspective in clinical medicine encompassing integrated physiology and molecular mechanisms. In conclusion, exercise improves microvascular health in subjects with increased CV risk leading to healthier ageing and eventually better CV outcomes. Reprogramming of DNA methylation on $p66^{Shc}$ gene promoter may represent a putative link whereby exercise protects against age-related oxidative stress. The entire mechanistic landscape remains to be addressed in future studies.

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Conflict of interest: none declared.

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SUPPLEMENTARY MATERIAL

Study design

In this randomized controlled trial, we investigated the influence of a 12-week high-intensity interval training (HIIT) on retinal microvascular phenotype as well as targeted DNA methylation of $p66^{Shc}$ and oxidative stress in ageing sedentary subjects with increased cardiovascular (CV) risk (SR) in a two parallel group design.

Anthropometric measurements, blood sampling and vascular imaging were performed in the morning under standardized fasting conditions. All examinations and the exercise training took place at the Department of Sport, Exercise and Health in Basel, Switzerland. This study was planned and conducted according to the Helsinki Declaration (World Medical Association, 2001) and the CONSORT guidelines[1]. The Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351) approved the study. All participants signed a written informed consent. The study has been registered at ClinicalTrials.gov (NCT02796976).

Inclusion and exclusion criteria

From January 2016 to December 2017 we recruited sedentary (≤ 3 METs/week) men and women aged 50–80 years with increased CV risk (SR) (≥ two CV risk factors). The risk factors were defined as hypertension (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic blood pressure (BP) during 24h monitoring or treatment with antihypertensive medications), body mass index (BMI) (≥ 30 kg/m^2), high fasting plasma glucose levels (≥ 5.6 mmol/l or antidiabetic medications), high triglyceride levels (> 1.7 mmol/l), low high-density lipoprotein levels (< 1.0 mmol/l (male); < 1.2 mmol/l (female)), high low-density lipoprotein levels (> 4.9 mmol/l or cholesterol lowering drugs), current smoking status (supplementary material online, Figure S2). Exclusion criteria were decompensated cardiopulmonary disease or chronic inflammatory disease, chronic eye disease or compromising orthopaedic problems.

Static Retinal Vessel Analysis (SVA)

Retinal vessel diameters were measured using the Retinal Vessel Analyzer (RVA, IMEDOS Systems, Jena, Germany) and a fundus camera (450 FF, Carl Zeiss, Jena, Germany). After pupil dilatation with conventional eye drops (Tropicamide 0.5%) and 20 minutes of rest, three valid images were taken from one eye at an angle of 50°. The detailed procedure has been described elsewhere[2]. Briefly, the

diameters of retinal arterioles and venules were analyzed semi-automatically in an area of 0.5-1 disc-diameter from the optic disc margin using the analyzing software (Vesselmap 3.0, Visualis, Imedos Systems UG). Diameters were averaged to central retinal arteriolar (CRAE) and central retinal venular (CRVE) equivalents using the Paar-Hubbard formula[3]. Vessel diameters are presented in µm, as one measuring unit of the imaging device relates to 1 µm in the model of Gullstrand's normal eye. The arteriolar-to-venular diameter ratio (AVR) was calculated from CRAE and CRVE. All fundus images were taken and analyzed by the same experienced investigator to eliminate interobserver variation. The investigator was blinded for group allocations. In our study, after re-analysis of 30 images, the correlation coefficient (CC) for CRAE was r =0.98, the coefficient of variation (CVar) was 8.30%. For CRVE, the CC was r =0.97 and the CVar was 6.27% (AVR: r =0.97 and CVar =9.84%), proving high reproducibility for all three retinal parameters (p <0.001 each).

Blood sampling

All blood samples were withdrawn by venipuncture of the cubital fossa of the right or left arm by trained medical staff in a fasting state. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood following classical density gradient separation protocol using Ficoll®-Paque (GE Healthcare Europe GmbH, Switzerland) and Leucosep™ 50ml tubes (Greiner BIO-ONE, Germany). Isolated PBMCs were washed with PBS, resuspended in 600µl RLT buffer (RNeasy Mini Kit, Qiagen, Switzerland) and stored at -80°C for subsequent analysis. All researchers performing further analysis of blood samples were blinded for group allocation.

3-nitrotyrosine plasma levels

Levels of oxidative stress marker 3-nitrotyrosine (3-NT) in plasma were measured in all participants using OxySelect™ Nitrotyrosine ELISA kit (Cell Biolabs, CA, USA) following the manufacturer`s instructions. Briefly, 50 µl of nitrated bovine serum albumin (BSA) standard and plasma samples were added to nitrated BSA pre-absorbed enzyme-inmmunoassay (EIA) plate and incubated for 10 min. Anti-nitrotyrosine antibody was added and incubated for one hour followed by the addition of horseradish peroxidase (HRP) conjugated secondary antibody. Warm substrate solution as well as stop solution were added and absorbance was immediately read at 450 nm using a spectrophotometer. Nitrotyrosine was determined by comparison with a standard curve prepared from predetermined nitrated BSA standards.

p66^{Shc} gene expression

Gene expression of p66^{Shc} was assessed before and after HIIT or standard physical activity (PA) recommendations in 20 subjects randomly selected from each group. The analysis of p66^{Shc} gene expression was performed in PBMCs by real-time quantitative polymerase chain reaction (RT-qPCR). RNA was extracted with Direct-Zol^{TM} RNA miniprep kit (Zymo research, CA, USA) and cDNA was synthesized with high capacity cDNA conversion kit (Applied Biosystems, Foster City, CA, USA). p66^{Shc} mRNA levels were detected by RT-qPCR using ABI 7900HT system (Applied Biosystems, Foster City, CA, USA) and FastStart Universal SYBR Green technology (Roche, Basel, Switzerland). Actin-Beta (ACTB) gene was used as endogenous control for normalizing RNA concentration. Differences in cycle threshold (Ct) values between test gene and endogenous control (ACTB; ΔCt) were calculated and used for statistical analysis.

DNA methylation analysis

DNA methylation analysis was performed in the same 20 subjects before and after HIIT or standard PA recommendations. Genomic DNA was purified from PBMCs using phenol:chloroform:isoamyl alcohol (Sigma Aldrich, St. Louis, USA), nucleospin Gel and PCR cleanup kit (Macherey-Nagel, PA, USA). Purified DNA (1µg) was used to assess DNA methylation of p66^{Shc} promoter. Briefly, methylated cytosines were captured with MethylMiner Enrichment Kit (Invitrogen, CA, USA) and level of methylation was assessed with promoter specific primers coupled with ABI 7900HT RT-qPCR system and fluorescence-based FastStart Universal SYBR Green technology (Roche, Basel, Switzerland). Methylated and non-methylated control duplexes provided by manufacturer were used as controls for methyl-CpG-binding-domain (MBD) capture. The amount of DNA pulled down by MBD protein was normalized to input (starting DNA material) of each sample. The primers used for detection of CpG islands in the p66^{Shc} promoter are indicated in supplementary material online, Table S4.

Anthropometry, physical fitness and activity

All anthropometric measurements were performed according to standard procedures as described in detail in our published study protocol[2]. Cardiorespiratory fitness was measured with an individualized treadmill ramp protocol as previously recommended[2, 4, 5]. We measured ventilatory parameters including maximal oxygen uptake (VO_2max) and maximal heart rate (HRmax) using the

Cortex Metalyzer R 3B metabolic test system (Cortex Biophysik GmbH, Leipzig, Germany). All participants wore an Aipermotion 440 accelerometer (Aipermon GmbH, Munich, Germany) on six consecutive days on their left hip. From the five most active days, we calculated total steps per day and minutes of walking per day using the AiperView 440 and ActiCoach MPAT2Viewer Software (Aipermon GmbH, Munich, Germany). This system has been extensively validated[6, 7]. The Freiburg Questionnaire of Physical Activity (FQPA) was used to assess self-reported sport activities[8] in metabolic equivalents (METs) per week based on the Ainsworth Compendium[8, 9]

Exercise intervention

SR group were randomized to the intervention or control group by blind drawing pieces of paper from an envelope by an independent research assistant. The physical exercise intervention for the SR group was a 12-week Nordic Walking-based and supervised HIIT performed three times per week. In the first week, the participants trained with an intensity of 75% of HRmax to get familiarized with a continuous walking training. In the second week, we performed a stepwise increase of the intensity with up to 80-90% of HRmax. In the following 10 weeks, the participants performed the HIIT based on the following protocol and with a total duration of 45 minutes per session: warm-up for 10 minutes at 60-70% HRmax followed by a high-intensity interval consisting of 4x4 minutes at 80-90% HRmax with 3min. of active recovery at 60-70% HRmax and a cool-down with 10 minutes at 60-70% HRmax. HR was monitored during training by standard heart rate sensors. The control group received PA recommendations based on the European Guidelines on Cardiovascular Disease Prevention in Clinical Practice[10].

Statistical analysis and sample size calculation

The primary outcome was the effect of exercise training on AVR in the HIIT group compared to the control group. We used mean and standard deviation to describe baseline characteristics and effects of exercise training and control condition. To visualize primary and secondary outcomes we used boxplots. We used analysis of covariance to compare differences in primary and further outcomes between the control and the intervention group corrected for the baseline value and main confounders[11]. We calculated t-tests for dependent samples to describe differences in the HIIT group and control group separately. All statistical tests with a 2-sided confidence interval of 95% were calculated using R version 3.5.0. For the intension to treat analysis we used multiple

imputation by chained equations using the “mice” package (version 3.3.0) in R (version 3.5.2) to impute the missing data[12, 13]. Specifically, we imputed 320 datasets with 10 iterations each. Convergence was assessed graphically. We applied predictive mean matching for imputation of continuous variables and logistic regression for binary variables. The results from the regression models based on the imputed datasets were pooled using Barnard-Rubin adjusted degrees of freedom for small samples[14].

For the sample size calculation, we assumed an expected difference in AVR of 0.05 with a standard deviation of 0.05 in SR after 12 weeks between intervention and control group[15]. Thus, a total sample size of 68 participants in the SR group was needed to reach a target power of 80% with a 2-sided significance level of 0.05. POWER and GLMPOWER procedures in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) were used to perform the sample size calculations.

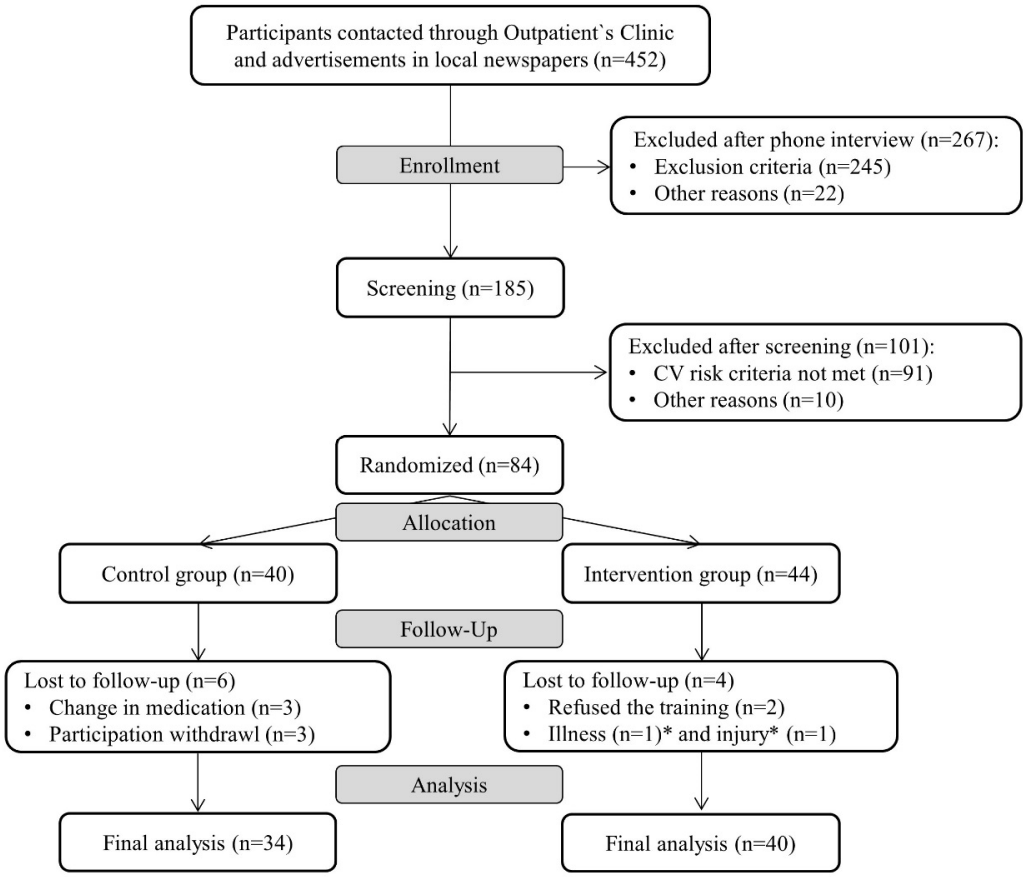

Figure S1. Flow-chart

*unrelated to exercise training.

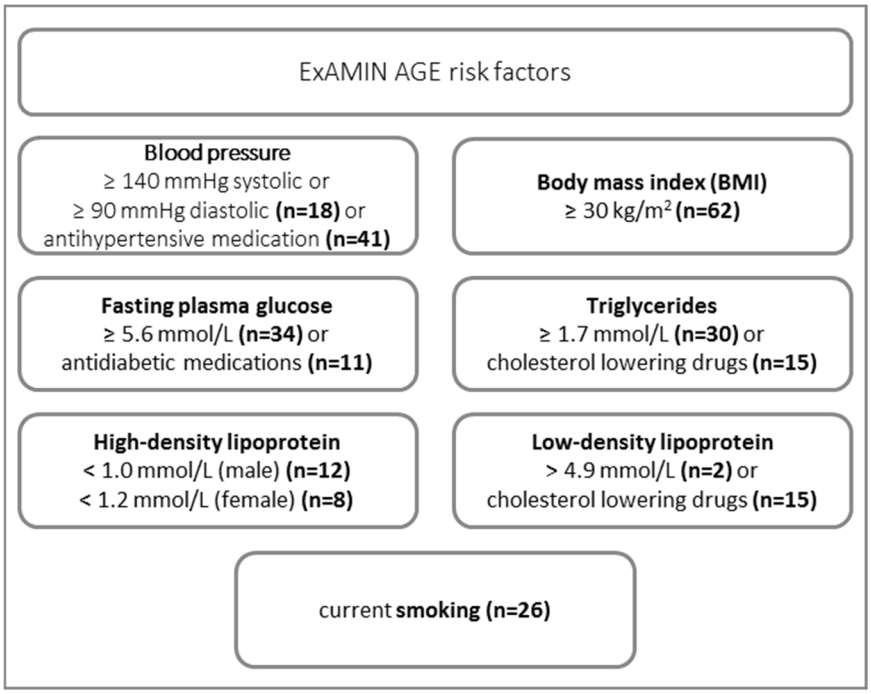

Figure S2. Definition and distribution of CV risk factors in the EXAMIN AGE Study.

SUPPLEMENTARY TABLES

Table S1. Population characteristics in sedentary at risk before and after intervention.

	SR intervention group (n=40) mean (SD)			**SR control group (n=34) mean (SD)**		
	pre	post	p	pre	post	p
Anthropometry data						
Age (years)	59 (6)			58 (7)		
Sex (f/m)	18/22			20/14		
Weight (kg)	94.3 (12.7)	93.14 (12.7)	0.003	93.2 (14.5)	92.4 (14.2)	0.012
BMI (kg/m^2)	32.9 (3.3)	32.5 (3.4)	0.008	32.9 (4.8)	32.6 (4.7)	0.085
WC (cm)	110 (9)	108 (10)	0.032	111 (13)	110 (13)	0.812
Fat mass (kg)	36.6 (8.8)	34.6 (9.1)	0.000	38.9 (10.7)	38.7 (10.1)	0.768
Muscle mass (kg)	32.1 (7.1)	32.7 (7.0)	0.016	30.1 (6.1)	29.8 (5.5)	0.328
Rest systolic BP (mmHg)	134 (14)	134 (12)	0.854	129 (14)	131 (15)	0.205
Rest diastolic BP (mmHg)	88 (10)	90 (7)	0.318	85 (10)	84 (10)	0.599
24h. systolic BP (mmHg)	130 (11)	133 (12)	0.179	129 (9)	125 (11)	0.154
24h. diastolic BP (mmHg)	82 (7)	83 (8)	0.236	79 (8)	78 (10)	0.799
Fasting glucose (mmol/l)	5.8 (2.1)	5.7 (1.7)	0.177	5.8 (1.4)	5.6 (1.2)	0.320
Triglyceride (mmol/l)	1.8 (1.0)	1.8 (1.1)	0.526	1.6 (0.8)	1.8 (1.0)	0.098
HDL (mmol/l)	1.3 (0.3)	1.3 (0.3)	0.487	1.4 (0.3)	1.4 (0.4)	0.781
LDL (mmol/l)	3.3 (0.8)	3.0 (0.8)	<0.001	3.0 (0.7)	2.9 (0.8)	0.268
Activity and fitness						
FQPA (METs)	1.6 (3.3)	23.1 (18.0)	<0.001	0.9 (2.1)	1.8 (4.2)	0.056
Steps per day (n)	8591 (3628)	9064 (3497)	0.423	9255 (3648)	8920 (4108)	0.455
Walking per day (min)	103 (44)	109 (43)	0.405	111 (42)	108 (47)	0.534
VO_2max (ml/min/kg)	26.4 (3.8)	28.7 (4.0)	<0.001	26.1 (5.0)	25.0 (4.0)	0.004

Retinal vessel diameter						
CRAE (µm)	175 (14)	181 (13)	0.001	168 (14)	170 (16)	0.108
CRVE (µm)	222 (14)	220 (14)	0.007	214 (17)	214 (17)	0.255
AVR	0.79 (0.04)	0.82 (0.05)	<0.001	0.79 (0.05)	0.79 (0.05)	0.494

Abbreviations: SR, sedentary at risk; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein; VO2max, maximal oxygen uptake; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; SD, standard deviation; p, level of significance pre- to post-intervention.

Table S2. Adjusted group differences of retinal vessel diameters, $p66^{Shc}$ expression and oxidative stress in sedentary at risk before and after intervention.

		CRAE (µm)		**CRVE (µm)**		**AVR**		**ΔΔCt($p66^{Shc}$/ ACTB) (AU)**		**3-NT (µg/mL)**	
	Model	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
HIIT vs. CC	1	5.02 (2.20;7.85)	<0.001	-3.26 (-6.13;-0.39)	0.027	0.03 (0.02;0.04)	<0.001	-5.31 (-10.27;-0.34)	0.037	-2.45 (-3.96;-0.94)	0.002
	2	5.04 (2.17;7.91)	<0.001	-3.39 (-6.30;-0.49)	0.023	0.03 (0.03;0.04)	<0.001	-5.15 (-10.17;-0.12)	0.045	-2.25 (-3.74;-0.77)	0.003
	3	5.59 (2.66;8.52)	<0.001	-2.64 (-5.61;0.33)	0.080	0.04 (0.03;0.05)	<0.001	-5.23 (-10.52;0.05)	0.052	-2.35 (-3.81;-0.90)	0.002
	4	4.13 (0.47;7.79)	0.024	-3.07 (-7.74;1.59)	0.189	0.03 (0.02;0.05)	<0.001	-6.05 (-12.02;-0.09)	0.047	-4.01 (-6.31;-1.72)	0.001
	5	1.98 (-2.50;6.47)	0.373	-4.47 (-10.39;1.45)	0.134	0.03 (0.01;0.05)	0.008	-7.46 (-14.80;-0.12)	0.047	-4.28 (-7.31;-1.24)	0.007

Abbreviations: CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; Ct , cycle threshold; ACTB, actin beta; AU, arbitrary units; 3-NT, 3-nitrotyrosine; HIIT, high-intensity interval training (n=40); CC, control condition (n=34); CI, confidence interval; p, level of significance for the regression. Model 1 adjusted for age; Model 2 adjusted for age and delta body mass index (ΔBMI). Model 3 adjusted for age, ΔBMI, systolic and diastolic blood pressure. Model 4 adjusted for age, ΔBMI, systolic and diastolic blood pressure and cardiovascular (CV) medication; Model 5 adjusted for age, ΔBMI, systolic and diastolic blood pressure, CV medication and Δ maximal oxygen uptake.

Table S3. Intension to treat analysis of retinal vessel diameters in sedentary at risk before and after intervention.

		CRAE (µm)		**CRVE (µm)**		**AVR**	
	Model	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
	1	5.46 (1.19;9.73)	0.013	-1.54 (-6.17;3.08)	0.506	0.03 (0.01;0.05)	<0.001
HIIT	2	5.56 (1.41;9.71)	0.009	-1.55 (-6.20;3.10)	0.505	0.03 (0.01;0.05)	<0.001
vs.	3	6.44 (1.76;11.12)	0.008	-0.67 (-5.76;4.42)	0.793	0.03 (0.01;0.05)	0.002
CC	4	6.43 (1.72;11.13)	0.009	-0.59 (-5.73;4.55)	0.818	0.03 (0.01;0.05)	0.002
	5	4.54 (-2.06;11.14)	0.169	-1.38 (-8.70;5.93)	0.703	0.03 (0.00;0.06)	0.049

Abbreviations: CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; HIIT, high-intensity interval training (n=40); CC, control condition (n=34); CI, confidence interval; p, level of significance for the regression. Model 1 adjusted for age; Model 2 adjusted for age and delta body mass index (ΔBMI). Model 3 adjusted for age, ΔBMI, systolic and diastolic blood pressure. Model 4 adjusted for age, ΔBMI, systolic and diastolic blood pressure and cardiovascular (CV) medication; Model 5 adjusted for age, ΔBMI, systolic and diastolic blood pressure, CV medication and Δ maximal oxygen uptake.

Table S4. Primers used in the EXAMIN AGE Study.

Primer Name	Sequence (5'-3')
ACTB_FRT	GTTGTCGACGACGAGCG
ACTB_RRT	GCACAGAGCCTCGCCTT
$p66^{Shc}$_FRT	CTGACACTTTCAAAGCGGTG
$p66^{Shc}$_RRT	GTATGTGCTCACTGGCTTGC
$p66^{Shc}$_CpGisland1_F	TCTACCTCAGGGTCCCTCCT
$p66^{Shc}$_CpGisland1_R	AGCCTCCGATTGGCTTAGAT
$p66^{Shc}$_CpGisland2_F	GGACGCGAACTTCAGACTTC
$p66^{Shc}$_CpGisland2_R	CAACGATCCTCGGCTAACTC
$p66^{Shc}$_CpGisland3_F	GGAGTTTCAGGGATTGACGA
$p66^{Shc}$_CpGisland3_R	GCCCAGAAGTCTGAAGTTCG

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4. Publication 3: Retinal endothelial function in cardiovascular risk patients: a randomized controlled exercise trial

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Abstract

The aim of this study was to investigate, for the first time, the effects of high-intensity interval training (HIIT) on retinal microvascular endothelial function in cardiovascular (CV) risk patients. In the randomized controlled trial, 84 middle-aged and previously sedentary patients with increased CV risk (aged 58±6 years) with ≥two CV risk factors were randomized into a 12-week HIIT (n=44) or control group (CG, n=40) with standard physical activity recommendations. A blinded examiner measured retinal endothelial function by flicker light-induced maximal arteriolar (ADmax) and venular (VDmax) dilatation as well as the area under the arteriolar (AFarea) and venular (VFarea) flicker curve using a retinal vessel analyzer. Standardized assessments of CV risk factors, cardiorespiratory fitness and retinal endothelial function were performed before and after HIIT. HIIT reduced body mass index, fat mass, low-density lipoprotein and increased muscle mass and maximal oxygen uptake (VO2peak). Both ADmax (pre: 2.7±2.1%, post: 3.0±2.2%, p=0.018) and AFarea (pre: 32.6±28.4%*s, post: 37.7±30.6%*s, p=0.016) increased after HIIT compared to CG (ADmax, pre: 3.2±1.8%, post: 2.9±1.8%, p=0.254; AFarea, pre: 41.6±28.5%*s, post: 37.8±27.0%*s, p=0.186). Venular function remained unchanged after HIIT. There was a significant association between Δ-change VO2peak and Δ-changes ADmax and AFarea (p=0.026, R^2=0.073; p=0.019, R^2=0.081, respectively). 12-weeks of HIIT improved retinal endothelial function in middle-aged patients with increased CV risk independent of the reduction of classical CV risk factors. Exercise has the potential to reverse or at least postpone progression of small vessel disease in older adults with increased CV risk under standard medication. Dynamic retinal vessel analysis seems to be a sensitive tool to detect treatment effects of exercise interventions on retinal microvascular endothelial function in middle-aged individuals with increased CV risk.

Introduction

Cardiovascular (CV) disease remains to be the main health care burden in western countries. Microvascular endothelial dysfunction is a key driver for CV disease progression[1]. It is estimated that 90% of endothelial cells are located in the microcirculation. The retinal microcirculation is an accessible vascular bed for the assessment of microvascular function[2, 3]. It shares the embryological origin and morphological as well as physiological properties with the cerebral circulation and is considered as a marker of cerebrovascular disease. Analysis of static retinal vessel diameters enhances the accuracy of CV diagnosis, documented by a 21% and 10.1% reclassification rate for CV events[4] and stroke[5]. Narrower arteriolar and wider venular diameters are associated with an increased risk of coronary heart disease[6], stroke[7] as well as CV mortality[8]. Although retinal vessel diameters are key regulators of microvascular blood flow, they do not reflect microvascular endothelial function per se. Dynamic retinal vessel analysis (DVA) is a new diagnostic tool for the assessment of microvascular endothelial function by flicker light-induced retinal vessel vasodilatation over time[9]. Few studies exist that investigated the association of flicker light-induced dilatation and CV disease. The Maastricht Study, a large population-based cohort study, found a reduced retinal arteriolar flicker response in patients with pre-diabetes, which was further blunted in patients with type 2 diabetes compared to patients with a normal glucose metabolism[10]. A reduction of the retinal arteriolar flicker response has been shown in patients with increased CV disease risk, with further reductions in the presence of chronic heart failure[11]. In the same cohort flow-mediated vasodilatation (FMD), as the gold standard for endothelial function in the macrocirculation, did not distinguish between healthy controls, patients with increased CV risk and heart failure.[11] There is a significant but only moderate correlation between large artery FMD and retinal microvascular endothelial function[11, 12]. The retinal flicker response is a new non-invasive microvascular biomarker for cardiovascular risk and has been described as a window to the heart[2].

High levels of physical activity have been associated with a reduction of CV disease risk and a lower incidence of stroke and coronary heart disease[13]. High-intensity interval training (HIIT) has been shown to not only increase cardiorespiratory fitness but also to efficiently reduce CV disease risk and improve vascular function, comparable[14] or even more effective than moderate continuous training[15, 16]. The low risk for adverse events during HIIT in CV risk

patients has been shown to be comparable to moderate continuous training[17]. Our study was designed to investigate, for the first time, the effects of HIIT on retinal microvascular endothelial function in patients with increased CV risk from the EXAMIN AGE cohort[18].

Methods

Design and study population

The present randomized controlled trial investigated the effects of HIIT on retinal endothelial function in patients with increased CV risk. Patients were randomized into a 12-week HIIT or physical activity recommendations according to current guidelines[19] as control condition. Recruitment was performed from January 2016 to December 2017 through our Outpatient`s Sports Medicine and Prevention Clinic or advertisements in local newspapers in and around the City of Basel. An independent and blinded research assistant drew group allocation from an envelope to performed randomization after baseline assessments. Anthropometric measurements and dynamic retinal vessel analysis were performed in the morning under fasting conditions. Appointments took place at the Department of Sport, Exercise and Health in Basel, Switzerland. This study was approved by the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351) and conducted according to the Helsinki Declaration (World Medical Association, 2001) and the CONSORT Guidelines. All participants signed a written informed consent before the first measurement took place. The study was registered at ClinicalTrials.gov (NCT02796976).

Inclusion and exclusion criteria

Previously sedentary men and women aged 50-80 years with increased CV risk were included. Participants needed to have at least two of the CV risk factors described in Table 1. Exclusion criteria were decompensated cardiopulmonary disease, chronic inflammatory disease, chronic eye diseases or compromising orthopaedic problems.

Table 1. Definition and distribution of risk factors.

Risk factor	Definition	HIIT	CG
		n	n
High body mass index	≥ 30 kg/m^2	33	24
Hypertension	≥ 140 mmHg sys. or ≥ 90 mmHg dia. BP during 24h monitoring or	9	3
	treatment with antihypertensive medications	18	18
Diabetes	fasting glucose ≥ 5.6 mmol/l or	15	16
	antidiabetic medications	5	6
Hypertriglyceridemia	Triglyceride > 1.7 mmol/l	9	14
Smoking	current smoking status	11	11
Low HDL	< 1.0 mmol/l (male); < 1.2 mmol/l (female)	9	4
High LDL	> 4.9 mmol/l or	4	0
	cholesterol lowering drugs	5	8

Abbreviations: HIIT, high-intensity interval training group; CG, control group; sys, systolic; dia, diastolic; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Dynamic retinal vessel analysis

We measured the right eye following standardized procedures after pupil dilatation with Tropicamide 0.5% SDU Faure (THEA Pharma, Schaffhausen, Switzerland) using the retinal vessel analyzer system (IMEDOS Systems Ltd., Jena, Germany) and a fundus camera (FF450; Carl Zeiss Ltd., Jena, Germany). In six patients, we measured the left eye due to local eye diseases on the right eye. All measurements took place in a quiet, dark and temperature-controlled room (20-22°C). To measure the region of interest, we used a fixation needle that additionally reduced eye movements of the patients. In the upper temporal quadrant, a straight arteriolar and venular segment one optic disc diameter away from the optic disc edge were marked for continuous diameter recording (Figure 2a). Identical segments were measured pre- and post-intervention in each patient. The total duration of retinal vessel recording was 350 seconds, divided into a 50 seconds baseline period and three cycles of 20 seconds flicker light stimulus followed by 80 seconds recovery. Raw data of all three cycles were combined and averaged to analyze maximal arteriolar (ADmax) and venular (VDmax) flicker response and area under the arteriolar (AFarea) and venular (VFarea) flicker curve in response to the resting value during the first 50 seconds. Two blinded and experienced

scientists independently judged the quality of every signal as previously described[20]. Only signals with sufficient quality were included in the final analysis. The intra-class correlation coefficient for the same protocol of flicker light-induced retinal arteriolar dilatation has previously been reported to be 0.82[21].

Patients` characteristics

Standard procedures of anthropometric measurements were performed according to the description in our published study protocol[18]. Blood pressure (BP) was measured twice after ten minutes of rest with the automatic BP monitor system (Omron Healthcare, Germany) just before the retinal analysis and in a 24h monitoring using an oscillometric cuff-based sphygmomanometer on the right arm (Mobil-O-Graph®, I.E.M GmbH, Germany). Mean arterial pressure (MAP) after 10 minutes of rest was calculated with the following formula: MAP = [2 × rest diastolic blood pressure + rest systolic blood pressure] / 3. An individualized treadmill ramp protocol was used to analyse cardiorespiratory fitness as previously recommended[18, 22, 23]. Briefly, the speed or incline of the treadmill were increased every minute until individual exhaustion. Two independent sport scientist inspected individual exhaustion criteria by evaluation of maximal heart rate (HRmax), respiratory exchange ratio (RER), VO2 levelling off, Borg scale, blood lactate as well as blood pressure immediately after termination of the exercise test. Three out of five parameters needed to be fulfilled. In seven patients, the cardiorespiratory exercise test had to be repeated because exhaustion criteria were not met. We measured maximal heart rate and ventilator parameters including VO2peak using the Cortex Metalyzer R 3B metabolic test system (Cortex Biophysik Ltd, Leipzig, Germany).

Exercise intervention and control condition

Patients were randomized into a 12-week HIIT group, three times per week, or a control group (CG) after the last baseline assessment. The intervention groups were trained and supervised by experienced sport scientists. Three sport scientist supervised a group of 10 participants. Standard heart rate sensors measured participant`s heart rate during the training sessions. Sport scientists controlled the heart rate of each participant during and after every training

session. The first two weeks of the HIIT training included a technical orientated Nordic-Walking training with a stepwise increased intensity (75-90% of HRmax). In the following ten weeks, the HIIT groups performed a standardized protocol with a total duration of 45 minutes: warm-up at an intensity of 60-70% HRmax for ten minutes, 4x4 minutes at 80-90% HRmax with three minutes of active recovery in between at 60-70% HRmax and ten minutes cool-down at 60-70% HRmax. Participants needed to successfully complete 80% of the sessions during the 12-week intervention. We defined a session as completed if the patients were in a HR zone between 80-90% for at least 16 minutes with three clear recovery phase in between. The CG received PA recommendations based on the European Guidelines on Cardiovascular Disease Prevention in Clinical Practice.[19] Our recommendations were to be active for at least 30min/day up to 5 days/week in a moderate intensity or 15min/day for 5 days/week in a vigorous intensity.

Statistical analysis and sample size calculation

Primary outcome for the dynamic retinal vessel analysis was the change in maximal flicker induced arteriolar dilatation (ADmax) in response to HIIT compared to CG. We used mean and standard deviation to describe baseline and follow-up characteristics. A multiple linear regression model was used to describe the relationship between the delta change in microvascular responses and the delta change of classical risk factors such as body weight, body mass index (BMI), waist circumference, fat mass, VO2peak, systolic and diastolic BP, fating glucose levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and high sensitivity C-reactive protein (Hs-CRP). We calculated paired sample t-tests to describe differences in the HIIT group and CG separately. To analyse possible intervention effects, we used an ANCOVA to correct for the individual baseline value of each parameter. Additionally we calculated an ANCOVA for the retinal flicker response parameter (ADmax, VDmax, AFarea and VFarea) corrected for baseline as well as for age, sex, change (Δ) BMI, MAP and ΔVO2peak.[24] We used the MAP measured directly before the dynamic retinal vessel analysis because the actual BP affects retinal endothelial function directly.[25] All statistical tests were two-sided and used a significance level of 0.05. The Graph was generated in Excel 2016 based

on the median flicker response calculated for every second separately in the intervention group. All statistical tests were performed with R version 3.5.0.

To date, no studies on the effects of exercise on retinal microvascular endothelial function exist. Therefore, we used our previous data on the effects of a 10-week exercise intervention on static retinal microvascular diameters to estimate our sample size. Changes in retinal vessel diameter reflect structural and functional adaptations and endothelial function is thought to contribute to changes in retinal vessel diameter[26]. We found significant retinal arteriolar widening in a group of 15 obese younger individuals (aged 40±6 years) after 10 weeks of moderate-to-high intensity continuous endurance training[26]. Taking these results and possible dropouts into account, we conservatively estimated our sample size and planned to include 40 participants in each group to assess the effects of HIIT on retinal endothelial function. We additionally calculated the sample size based on a previous publication of our group in patients with metabolic syndrome compared to healthy normal weight subjects.[27] Based on this group difference we assumed a difference in mean maximal arteriolar dilation between the HIIT and CG group of 1.2% with a standard deviation of 1.8% after 12 weeks. To reach a target power of 80% with a 2-sided significance level of 0.05, we needed a total sample size of 74 participants. Taken dropouts into account, we aimed to include 40 patients in each group. We used G*Power software 3.1.9.2 for the sample size calculation.[28]

Results

Population characteristics

After screening for inclusion and exclusion criteria, 84 patients were randomized into HIIT or CG. The follow-up assessment was performed in 74 patients. Thirty-three controls and 36 patients undergoing HIIT reached sufficient video quality and were included in the final analysis (Figure 1). All participants included in the final analysis completed at least 80% of the training sessions with a sufficient intensity as described above. Two patients refused to perform the training and two patients dropped out due to illness or injury during the training phase not related to the intervention (Figure 1).

The distribution of risk factors is listed in Table 1. Seven patients in the intervention group had two, eleven patients had three, eleven patients had four and seven patients had ≥five CV risk factors. Seven patients in the control group had two, 13 patients had three, four patients had four and nine had ≥five CV risk factors. Detailed baseline characteristics of both groups

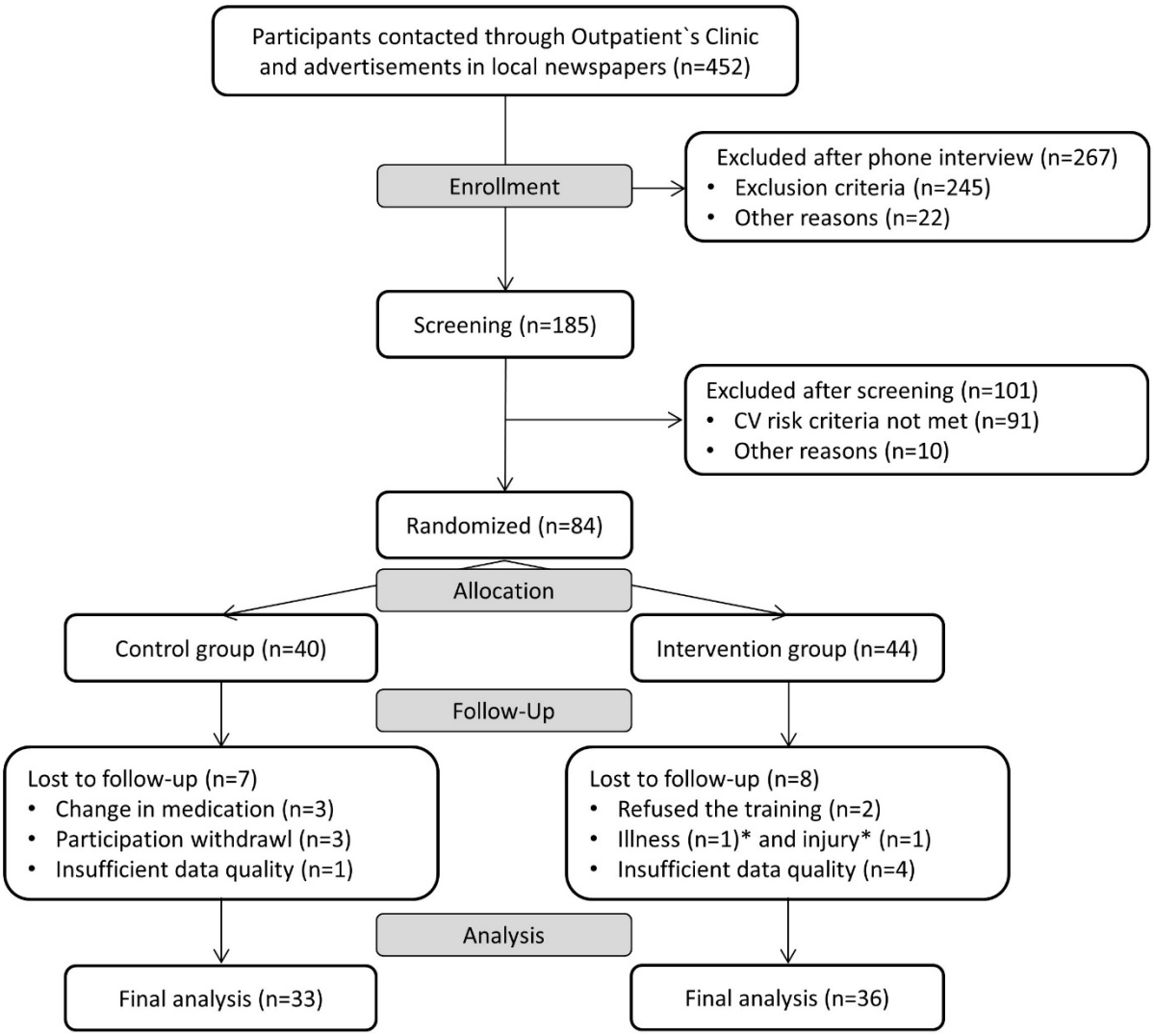

Figure 1. Flow-chart

*unrelated to exercise training.

at baseline as well as group alterations after the intervention period are presented in Table 2. Both groups reduced weight but only the patients undergoing HIIT reduced BMI, fat mass, LDL, increased muscle mass, physical activity (FQPA) and VO2peak. Triglycerides increased and VO2peak decreased in the CG during the 12 weeks (Table 2). No HIIT-related adverse events were observed.

Table 2. Population characteristics in patients with increased CV risk before and after exercise or control condition.

	Intervention group (n=36) mean (SD)			**Control group (n=33) mean (SD)**			
	pre	post	p[a]	pre	post	p[b]	p[c]
Patients` characteristics							
Age (years)	58 (5)			58 (7)			
Sex (f/m)	(16/20)			(19/14)			
Weight (kg)	95.6 (12.3)	94.3 (12.5)	0.003	92.9 (14.6)	92.1 (14.3)	0.011	0.440
BMI (kg/m^2)	33.3 (3.2)	32.8 (3.4)	0.007	32.7 (4.8)	32.5 (4.7)	0.085	0.266
WC (cm)	111 (9)	109 (11)	0.051	110 (13)	110 (13)	0.085	0.271
Fat mass (kg)	36.8 (8.5)	35.1 (8.8)	0.000	38.4 (10.5)	38.3 (10.0)	0.768	0.027
Muscle mass (kg)	32.8 (7.2)	33.2 (7.2)	0.037	30.2 (6.2)	29.8 (5.6)	0.328	0.026
VO2peak (ml/min/kg)	26.6 (3.8)	28.7 (4.1)	<0.001	26.3 (4.9)	25.2 (4.0)	0.002	<0.001
FQPA (METs)	1.2 (2.3)	23.6 (18.7)	<0.001	0.9 (2.2)	1.9 (4.2)	0.056	<0.001
Rest systolic BP (mmHg)	133 (14)	133 (12)	0.851	129 (14)	131 (15)	0.302	0.970
Rest diastolic BP (mmHg)	89 (10)	87 (7)	0.326	85 (10)	84 (10)	0.650	0.427
MAP(mmHg)	103 (9)	103 (8)	0.553	100 (10)	100 (11)	0.945	0.693
24h. systolic BP (mmHg)	129 (10)	132 (12)	0.147	127 (9)	125 (11)	0.286	0.057
24h. diastolic BP (mmHg)	82 (7)	83 (8)	0.232	78 (8)	78 (9)	0.776	0.281
Fasting glucose (mmol/l)	5.9 (2.2)	5.7 (1.8)	0.202	5.8 (1.5)	5.6 (1.2)	0.349	0.899
Triglyceride (mmol/l)	1.8 (1.1)	1.9 (1.2)	0.451	1.5 (0.7)	1.8 (1.0)	0.035	0.136
HDL (mmol/l)	1.3 (0.3)	1.3 (0.3)	0.547	1.4 (0.3)	1.4 (0.4)	0.755	0.731
LDL (mmol/l)	3.3 (0.9)	3.1 (0.9)	<0.001	3.0 (0.7)	2.9 (0.8)	0.447	0.079
Hs-CRP (mg/l)	3.43 (2.5)	3.07 (2.2)	0.197	3.93 (5.9)	3.05 (3.4)	0.135	0.379

Retinal flicker response							
ADmax(%)	2.7 (2.1)	3.0 (2.2)	0.027	3.2 (1.8)	2.9 (1.8)	0.254	0.036
VDmax(%)	4.0 (1.6)	3.8 (1.6)	0.355	4.4 (2.8)	4.0 (2.0)	0.132	0.863
AFarea (%*s)	32.6 (28.4)	37.7 (30.6)	0.034	41.6 (28.5)	37.8 (27.0)	0.186	0.034
VFarea (%*s)	43.4 (21.1)	41.7 (20.2)	0.545	48.6 (31.6)	42.2 (26.2)	0.060	0.415

Abbreviations: BMI, body mass index; WC, waist circumference; VO2peak, maximal oxygen uptake; FQPA, Freiburg Questionnaire of Physical Activity; METs, metabolic equivalents; BP, blood pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Hs-CRP, high sensitivity C-reactive protein; ADmax, maximal arteriolar dilatation; VDmax, maximal venular dilatation; AFarea, integral under arteriolar flicker curve; VFarea, integral under venular flicker curve; SD, standard deviation; p^a, p-value for paired sample t-tests pre- to post-intervention; p^b, p-value for paired sample t-tests pre- to post-control condition; p^c, p-value for the intervention effect analyzed by ANCOVA corrected for baseline.

Retinal microvascular flicker response

At baseline, retinal flicker response did not differ between the two randomized groups. ADmax and AFarea increased after HIIT compared to CG (Figure 2b), even after adjustment for the value at baseline, age, sex, Δ-change BMI and MAP (Table 3). Improvements of ADmax and AFarea were dependent on Δ-change in VO2peak (Table 3). There was a significant association between Δ-change VO2peak and Δ-change ADmax ($F(1, 66)=5.224$, $p=0.0255$) and AFarea ($F(1, 66)=5.836$, $p=0.018$). One ml/min/kg increase in VO2peak was associated with a 0.12% increase in ADmax as well as a 1.9%*s increase of AFarea. Exercise-related changes of classical risk factors such as reduction in body weight, BMI, fat mass and LDL were not statistically significantly associated with improvements of microvascular endothelial function ($p>0.05$ in all cases). In the CG, no changes in retinal flicker response parameters were observed (Figure 2c). Parameters of venular function (VDmax and VFarea) did not change after HIIT (Table 2 and 3).

Table 3. Adjusted models of retinal flicker light response in patients with cardiovascular disease after HIIT compared to control condition.

		ADmax (%)		**VDmax (%)**		**AFarea**		**VFarea**	
		β (95% CI)	p	β (95% CI)	p	β (95% CI)	p	β (95% CI)	p
	Model								
HIIT vs. CG	1	0.51 (0.05;0.95)	0.029	0.01 (-0.49;0.52)	0.955	7.92 (0.77;15.09)	0.031	2.79 (-4.64;10.22)	0.456
	2	0.55 (0.09;1.01)	0.020	0.10 (-0.41;0.61)	0.706	8.69 (1.50;15.88)	0.019	3.47 (-4.11;11.05)	0.363
	3	0.55 (0.10;1.01)	0.018	0.11 (-0.41;0.62)	0.680	8.59 (1.63;15.56)	0.016	3.67 (-3.92;11.26)	0.338
	4	0.14 (-0.49;0.77)	0.649	-0.39 (-1.10;0.32)	0.275	3.39 (-6.51;13.29)	0.495	-0.15 (-11.14;10.83)	0.978

Abbreviations: HIIT, high-intensity interval training; CG, control group; ADmax, maximal arteriolar dilatation; VDmax, maximal venular dilatation; AFarea, integral under arteriolar flicker curve; VFarea, integral under venular flicker curve; CI, confidence interval; p, level of significance for the regression models (ANCOVA). Model 1, adjusted for baseline, age and sex; Model 2, adjusted for baseline, age, sex and change (Δ) body mass index (BMI); Model 3, adjusted for baseline, age, sex, ΔBMI and mean arterial pressure (MAP); Model 4, adjusted for baseline, age, sex, ΔBMI, MAP and Δmaximal oxygen uptake.

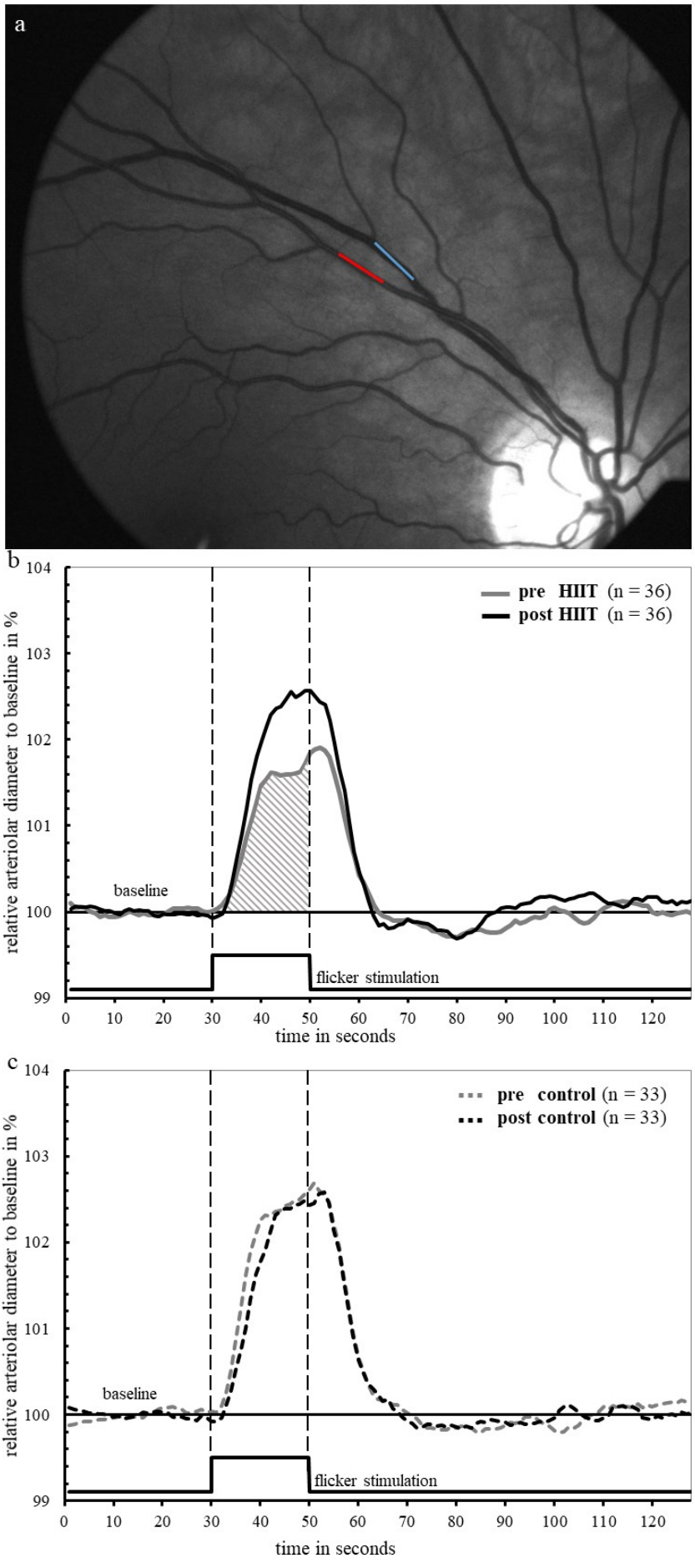
a
b
pre HIIT (n = 36)
post HIIT (n = 36)
relative arteriolar diameter to baseline in %
baseline
flicker stimulation
time in seconds
c
pre control (n = 33)
post control (n = 33)
relative arteriolar diameter to baseline in %
baseline
flicker stimulation
time in seconds

Figure 2. Fundus image and averaged retinal arteriolar dilatation in response to flicker light before and after HIIT

Detection of retinal arterioles and venules in the upper temporal quadrant before dynamic retinal vessel analysis (a). Retinal arteriolar dilatation in response to flicker light as % change compared to baseline in the intervention group (b) and the control group (c) before (pre) and after (post) HIIT or control condition.

Discussion

The present study demonstrates for the first time that short-term HIIT exercise training has the potential to improve retinal microvascular endothelial function in older patients with increased CV risk. HIIT-induced amelioration of microvascular function was independent of improvements of classical risk factors. The effects of exercise on arteriolar endothelial function are likely to be multifactorial but improvements of cardiorespiratory fitness as measured by VO2peak contribute to improved microvascular health.

Exercise, risk factors and microvascular endothelial function

Previous studies have reported associations of higher body weight[27], hypertension[29] as well as hypercholesterolemia[30] with decreased retinal endothelial function. After HIIT, we observed a reduction in BMI, fat mass and LDL levels whereas blood pressure and other classical risk factors remain unchanged. Interestingly, the improvements of microvascular endothelial function after HIIT were independent of changes in BMI as well as classical risk factors at baseline but depend on changes in VO2peak (Table 3). Exercise-induced improvements of VO2peak were significantly associated with improvements of arteriolar endothelial function. Five percent of changes in ADmax were explained by the increase in VO2peak after HIIT. Our findings demonstrate that cardiorespiratory fitness is an important mediator of the amelioration of microvascular endothelial function in patients with increased CV risk. A relative short-term HIIT intervention of three months has beneficial effects on retinal endothelial function even in middle-aged patients. ADmax in CV disease risk cohorts using the same device and protocol has been previously reported to be in the range of 2.3-2.4%[11, 31], which is comparable to our findings. The prevalence of CV risk factors was slightly

lower in our cohort and which might explain the disparity in ADmax compared to previous reports. ADmax in healthy and low risk individuals in a comparable age-range have been reported to be in the range of 3.6-3.8%[11, 32]. Our cohort showed an improvement of ADmax from 2.7% pre- to 3.0% post-exercise. The clinical relevance of this improvement needs to be verified in future studies.

Improvement of cardiorespiratory fitness rather than reduction of classic CV risk factors seems to play a key role for improvement of microvascular endothelial function after short-term exercise. Since classic risk factors have been associated with microvascular endothelial dysfunction in previous studies[10, 11, 27, 32-34], their reduction is likely to affect microvascular health long-term. However, we would like to speculate that unlike reduction of classic CV risk factors, HIIT-induced improvement of VO2peak has significant effects on retinal endothelial function. This is of high clinical relevance, since endothelial dysfunction in the macrocirculation has been shown to predict CV disease progression and event rates[35]. In patients at risk of coronary artery disease (CAD), endothelial dysfunction was associated with worse CV outcome[35]. It has also been argued that endothelial dysfunction may contribute to cerebral ischaemia and stroke[36]. However, the predictive value of retinal microvascular endothelial function for CV outcomes are not yet available. As a putative clinical perspective, our findings seem to strengthen recommendations for early exercise-based rehabilitation programs in patients at high risk, for example following CV events such as myocardial infarction or stroke. Improvement of cardiorespiratory fitness seems to induce immediate and direct effects on microvascular endothelial function before reduction of classic CV risk factors improve microvascular end-organ function. Dynamic retinal vessel imaging seems to be a valid diagnostic tool to detect microvascular endothelial function in patients at risk and to monitor lifestyle and potentially drug treatment effects.

Potential mechanisms

Exercise is a known modulator of nitric oxide (NO) bioavailability[37], which is considered to be the main local regulator of blood flow and endothelial function[38]. Increase in local blood flow during exercise is mediated by shear stress-induced production of NO. Higher intensities are thought to generate higher rates of shear stress and increased NO bioavailability[39]. Dorner et

al. showed that inhibition of NO can reduce retinal vessel dilatation in response to flicker light, proving NO dependence of retinal endothelial function[40]. NO blockage did not result in complete absence of dilation which suggests that other factors may also play a role. Nonetheless, the improvement of retinal microvascular endothelial function after HIIT can be explained, in large part, by an exercise-induced increase in NO bioavailability. Indeed, we have previously shown that regular exercise can decrease circulating levels of the NO inhibitor asymmetric dimethylarginine (ADMA) in obese subjects, which was associated with an increase in arteriolar diameters[26].

Another potential mechanism for an exercise-induced improvement of microvascular endothelial function is a reduction of inflammation in patients with CV disease. Exercise is known for its potential to reduce inflammation and improve CV outcome[41]. However, in our study, no reduction in high sensitivity C-reactive protein (hs-CRP) as a marker of systemic inflammation was observed. The low baseline levels of hs-CRP did not allow for significant changes after HIIT. The anti-inflammatory effects of HIIT on the improvement of retinal endothelial function could not be demonstrated in our study. Future investigations may have to include more specific inflammatory markers for further differentiation.

It remains to be discussed why exercise did not affect retinal venular dilatation. Venular wall shear stress has been shown to be much lower in post-capillary venular vessels compared to arterioles[42]. Much of the exercise-induced increase in shear stress is likely to be buffered by arterioles and the capillary system. Retinal venular vessels possess endothelial cells but only thin layers of smooth muscle cells, making it a more passive microvascular structure. Moreover, arterioles as the resistance vessels are predominantly affected by risk factors of arteriolosclerosis and microvascular remodelling compared to venules[43].

Dynamic retinal vessel analysis as a clinical diagnostic tool in cardiovascular prevention

DVA is a new non-invasive diagnostic tool that may help optimize CV risk stratification and monitoring of treatment effects on the microvascular target organ. Few previous studies have shown that dynamic retinal vessel analysis can differentiate between healthy controls and patients with heart failure[11], diabetes and pre-diabetes[10] as well as patients with metabolic syndrome[27] by quantifying retinal microvascular endothelial dysfunction. The reactive capacity of retinal arterioles to dilate in response to flicker light has been shown to be

predictive for the presence of heart failure and coronary artery disease[11, 31] and has been described as a window to the heart[2]. This is the first study to demonstrate that DVA can sensitively detect effects of exercise as an add-on treatment in patients already receiving medication for CV risk factors. In patients with CAD, immediate improvement of coronary endothelial function by short-term exercise, independent of achieving risk factor reduction, would have high potential to improve CV outcome. It remains to be shown whether exercise-induced improvements of retinal endothelial function does really reflect improvements of endothelial function in the coronary microcirculation. Moreover, prospective long-term follow up studies are warranted to determine the predictive value of retinal endothelial function for development of CV disease and for CV mortality.

Limitations

Some limitations apply to our study. We have investigated the short-term effects of HIIT on retinal endothelial function in older sedentary adults with increased CV risk as prove of principle. The effects of long-term exercise interventions need to be assessed in future studies. Moreover, our data cannot per se be generalized to younger or healthy populations. It would be of considerable scientific interest and clinical relevance to elucidate whether HITT is more effective than, for example, moderate continuous exercise training with respect to improvement of microvascular endothelial function. This will have to be examined in future studies and was beyond the scope of our current approach. Risk factors of our patients have been very well characterized and potential mechanisms have been discussed on the basis of the available data. While we have included Hs-CRP as a circulating inflammatory biomarker, future studies may need to focus on more specific molecular mechanisms that help explain the effects of exercise on microvascular endothelial function. No outcome data is currently available on the predictive value of retinal endothelial function for CV morbidity and mortality. Long-term prospective follow-up studies are warranted to show that exercise-induced improvements of retinal endothelial function have added value for CV risk stratification and better CV outcome.

Conclusions and perspectives

Dynamic retinal vessel analysis is a non-invasive measurement of in-vivo microvascular endothelial function[9]. Short-term HIIT can improve microvascular endothelial function independent of improvements in classical risk factors. Exercise in addition to medication in older patients with increased CV risk has the potential to postpone progression of microvascular dysfunction and the process of vascular aging. HIIT has the potential to restore microvascular endothelial function beyond improvement of classical risk factors. The amelioration of arteriolar function is associated with improvement of cardiorespiratory fitness. Exercise as add-on therapy for middle-aged patients with increased CV risk factors has the potential to reverse or at least postpone progression of microvascular dysfunction and the process of vascular aging.

Future studies need to determine whether exercise-induced improvements of retinal microvascular endothelial function in patients with CV disease are associated with better CV outcome. Dynamic retinal vessel analysis allows access to the cerebrovascular bed and seems to be a sensitive diagnostic tool to detect treatment effects of exercise interventions on retinal microvascular endothelial function in patients with increased CV risk.

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Conflict of interest

The authors have declared no conflicts of interest.

Founding information

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5. Publication 4: Retinal endothelial function, physical fitness and cardiovascular risk: a diagnostic challenge

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Retinal Endothelial Function, Physical Fitness and Cardiovascular Risk: A Diagnostic Challenge

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Introduction: Dynamic retinal vessel analysis (DVA) is a new non-invasive method to quantify microvascular endothelial dysfunction by flicker light-induced dilatation (FID). FID has been shown to be impaired in type 2 diabetes as well as heart failure. The aim of the study was to analyze FID in healthy active versus healthy sedentary and cardiovascular (CV) risk patients in addition to corresponding static vessel diameters.

Methods: Thirty-one healthy active (HA, mean age 60 ± 8 years), 33 healthy sedentary individuals (HS, 59 ± 7 years) and 76 sedentary patients with increased CV risk (SR, 58 ± 6 years) were included in this cross-sectional study. Group differences in CV risk factors and cardiorespiratory fitness, maximal arteriolar (ADmax) and venular (VDmax) dilatation as well as the arteriolar (AFarea) and venular (VFarea) area under the flicker curve were analyzed. The central retinal arteriolar and venular diameters were used to calculate the arteriolar-to-venular diameter ratio (AVR).

Results: HS [ADmax = 3.5 (2.1)%; AFarea = 48.2 (31.9)%*s] showed higher FID compared to SR [ADmax = 2.7 (1.8)%, $p = 0.021$; AFarea = 34.5 (26.5)%*s, $p = 0.006$] and HA [AFarea = 32.8 (23.1)%*s, $p = 0.029$]. HA and SR did not significantly differ. HA had a higher AVR (0.87 ± 0.05) compared to HS (0.83 ± 0.04, $p < 0.001$) with further deterioration in SR (0.79 ± 0.05, $p < 0.001$). Interestingly, 28 participants had impaired FID but normal AVR and 43 participants had normal FID but impaired AVR.

Discussion: FID can differentiate between sedentary low and high risk individuals. However, FID in healthy active persons (HA) seemed impaired with a concomitant higher AVR. We postulate that lower FID in HA may be explained by predilatated arterioles and a reduced dilatation reserve. We recommend combination of FID with analysis of retinal vessel diameters to differentiate functional non-responders from manifest microvascular endothelial dysfunction and, thereby, improve microvascular risk stratification in a personalized medicine approach.

Clinical Trial Registration: ClinicalTrials.gov: NCT02796976 (https://clinicaltrials.gov/ct2/show/NCT02796976).

Keywords: microcirculation, flicker light-induced dilatation, retinal vessel diameters, physical activity, cardiovascular disease

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INTRODUCTION

Dynamic retinal vessel analysis is a new non-invasive diagnostic tool to assess microvascular endothelial dysfunction. Flicker light-induced dilatation (FID) of arterioles seem to reflect cardiovascular (CV) risk at a subclinical stage. Nägele et al. showed that FID is reduced in patients with CV risk factors compared to healthy controls with further deterioration in heart failure patients (Nagele et al., 2018b). Sörensen et al. (2016) demonstrated reduced FID in patients with prediabetes compared to healthy individuals with further impairments in patients with manifest type 2 diabetes (Sörensen et al., 2016). Impaired FID has also been associated with hypertension (Machalinska et al., 2018), hypercholesterolemia (Nagele et al., 2018a), obesity (Kotliar et al., 2011) and higher age (Kneser et al., 2009). With respect to static retinal vessel diameters, narrower arterioles, wider venules and a resulting lower arterio-venous ratio (AVR) have been associated with incidence hypertension (Wang et al., 2003; Wong et al., 2004; Ikram et al., 2006b), stroke (Ikram et al., 2006a; McGeechan et al., 2009) and a higher CV morbidity and mortality (Wang et al., 2007; Seidelmann et al., 2016). No studies to date have combined dynamic and static retinal vessel analysis in an individual patient-orientated approach.

Physical inactivity is a major risk factor for development of non-communicable chronic diseases such as CV disease. Lower physical activity (PA) is associated with a higher CV mortality (Handschin and Spiegelman, 2008). Blair et al. (1995) demonstrated a mortality risk reduction of 44% in individuals who improved their lifestyle from unfit to fit compared to participants who remained unfit (Blair et al., 1995). Moderate PA of 90 min per week has been associated with a reduction of all-cause mortality by 14% (Wen et al., 2011). Age and physical fitness are known to affect microvascular function. Bioavailability of nitric oxide (NO), a key modulator of endothelial function, is higher in young physically active individuals compared to sedentary controls and has been associated with improved microvascular endothelial function in the skin (Franzoni et al., 2004). Favorable retinal vessel diameters have previously been associated with higher PA and fitness (Anuradha et al., 2011; Hanssen et al., 2011; Streese et al., 2019). To date, no data are available on the association of PA and fitness with retinal microvascular endothelial function in healthy individuals and in patients with CV disease.

The aims of the study were twofold. We aimed to compare FID in healthy active (HA) with healthy sedentary (HS) individuals to determine the impact of lifelong PA on retinal endothelial function. Moreover, we aimed to compare HS with sedentary individuals at increased CV risk (SR) to determine the impact of CV risk factors on FID. We hypothesized that HA would have aggravated FID whereas SR would present with a blunted microvascular response. Our study, for the first time, aimed to report individual retinal FID in relation to the corresponding vessel diameters by combining dynamic and static retinal vessel analysis.

Abbreviations: ACmax, maximal arteriolar constriction; ADmax, maximal arteriolar dilatation; AFarea, arteriolar area under the flicker curve; AVR, arteriolar-to-venular diameter ratio; DVA, dynamic retinal vessel analysis; FID, flicker light-induced dilatation; FQPA, Freiburg Questionnaire of physical activity; HA, healthy active individuals; HS, healthy sedentary individuals; NO, nitric oxide; PA, physical activity; SR, sedentary patients with increased cardiovascular risk; VCmax, maximal venular constriction; VDmax, maximal venular dilatation; VFarea, venular area under the flicker curve.

MATERIALS AND METHODS

Design and Study Population

Participants from the EXAMIN AGE cohort (Streese et al., 2018) were recruited from January 2016 till December 2017 through local sports and running clubs, advertisements in local newspapers in and around the city of Basel and through our Outpatient Prevention Clinic. All participants signed a written informed consent before the first measurement at the Department of Sport, Exercise and Health in Basel, Switzerland took place. Anthropometric measurements and retinal vessel analysis were performed in the morning under fasting conditions. This study was approved through the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351) and conducted according to the Helsinki Declaration (World Medical Association, 2013). The study is registered at ClinicalTrials.gov (NCT02796976).

Inclusion and Exclusion Criteria

Men and women aged 50–80 years were included in the study. Inclusion criteria for HA was an active lifestyle [>9 metabolic equivalents (METs)/week]. Inclusion criteria for HS and SR was a sedentary lifestyle (≤ 3 METs/week). Additionally, SR needed to have at least ≥ 2 CV risk factors as described in **Figure 1A**.

Exclusion criteria for HA and HS were any risk factor described in **Figure 1A**, macular degeneration, glaucoma or any eye disease or history of CV, pulmonary or chronic inflammatory diseases. Exclusion criteria for patients with increased CV risk were chronic eye disease, decompensated cardiopulmonary or chronic inflammatory disease and/or restricting orthopedic problems. Two sport scientists independently allocated participants to the active or sedentary groups or to exclude the subject on mutual grounds on the basis of PA history, self-reported freiburg questionnaire of physical activity (FQPA), accelerometer data and maximal oxygen uptake (VO2max).

Retinal Vessel Analysis

After pupil dilatation of the right eye (in 16 patients the left eye was measured due to local eye problems) with Tropicamide 0.5% SDU Faure (THEA Pharma, Schaffhausen, Switzerland) and 10 min of rest, retinal endothelial function was measured using the retinal vessel analyzer system (IMEDOS; GmbH, Jena, Germany) and a fundus camera (FF450; Carl Zeiss GmbH, Jena, Germany). The measurements took place in a quiet, dark and temperature-controlled room (20–22°C). To reduce eye movements and to measure the region of interest, a fixation needle was used. One straight arteriolar and venular segment in the upper temporal quadrant, one optic disk diameter away from the optic disk edge were marked. Diameters of these

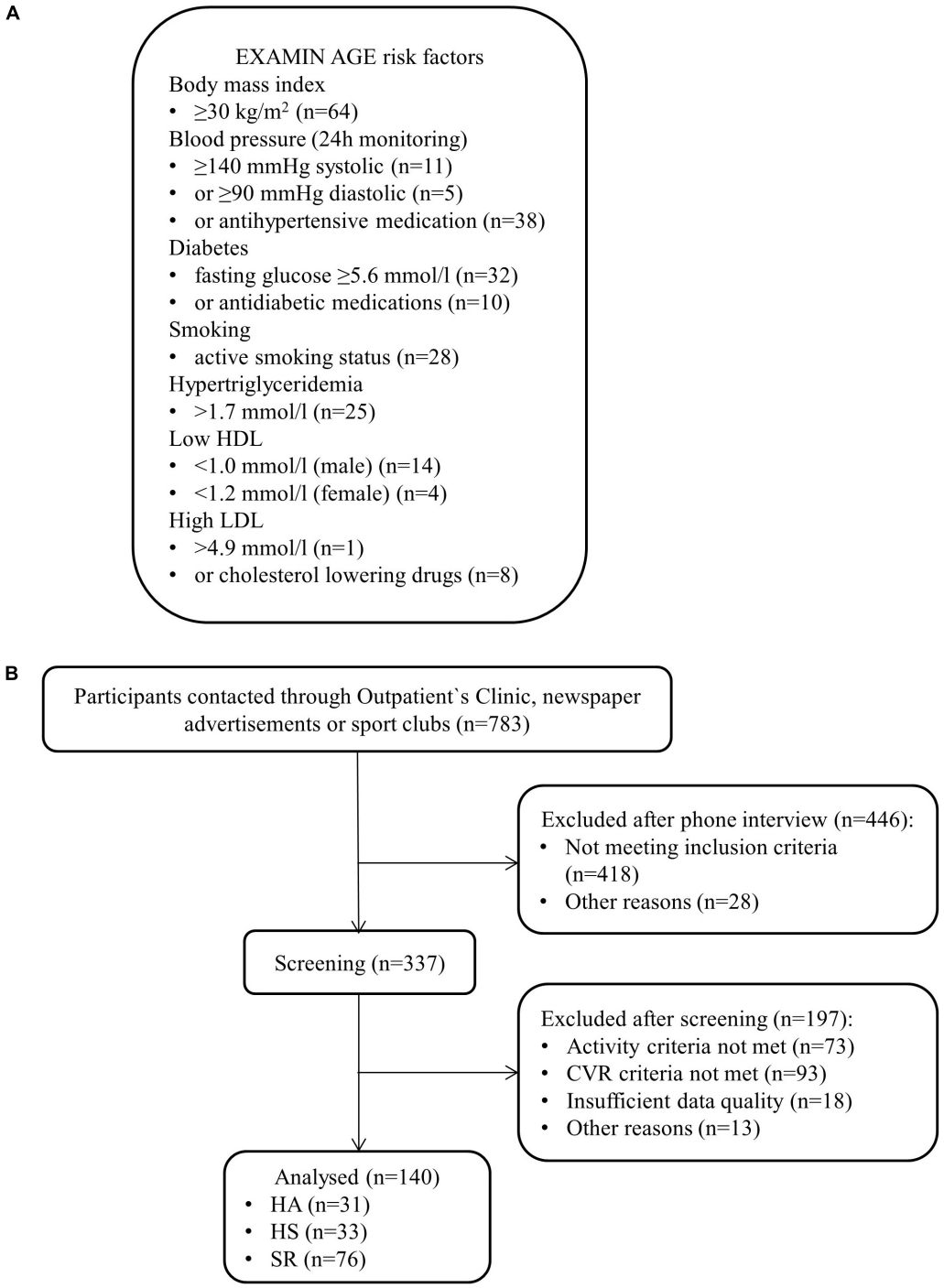

FIGURE 1 | Cardiovascular risk factors **(A)** and flow-chart **(B)**.

segments were continuously recorded for 350 s. The first 50 s (baseline) were followed by three cycles of 20 s flicker light (flicker frequency 12.5 Hz) followed by 80 s of recovery (green light without flicker). Based on the raw data we averaged the flicker cycles to calculate maximal arteriolar (ADmax) and venular (VDmax) flicker response, maximal arteriolar (ACmax) and venular (ACmax) constriction as well as the integral under the arteriolar (AFarea) and venular (VFarea) flicker curve. In order to improve the data quality, two experienced scientists independently judged the quality of every raw signal as previously described (Kotliar et al., 2017). Only raw signals with sufficient quality were included in the final analysis (**Figure 1B**). Al-Fiadh

previously reported an interclass correlation coefficient of 0.82 for the arteriolar dilatation using the same flicker protocol (Al-Fiadh et al., 2014). AVR was calculated from central retinal arteriolar (CRAE) and central retinal venular (CRVE) equivalents which were measured as previously described (Hanssen et al., 2011) using the Paar-Hubbard formula (Hubbard et al., 1999). Previous studies indicate that ADmax values below 2.5% (Al-Fiadh et al., 2015; Sörensen et al., 2016; Nagele et al., 2018b) as well as lower AVR values in the range of <0.82 (Wong et al., 2004; Ikram et al., 2006a) are associated with increased CV risk.

Anthropometry, Physical Fitness and Activity

All anthropometry measurements were performed as described in our published study protocol (Streese et al., 2018). Blood pressure (BP) was measured in a 24 h monitoring and twice before the microvascular assessments after 10 min of rest. Cardiorespiratory fitness including VO2max and maximal heart rate (HRmax) was measured according a treadmill ramp protocol as previously recommended (Bader et al., 1999; Myers and Bellin, 2000; Streese et al., 2018) using the Cortex Metalyzer R 3B metabolic test system (Cortex Biophysik GmbH, Leipzig, Germany). Participants wore an Aipermotion 440 accelerometer (Aipermon GmbH, Munich, Germany) on their left hip for six consecutive days to evaluate daily PA. Total steps per day and minutes of walking per day were calculated from the five most active days using AiperView 440 and ActiCoach MPAT2Viewer Software (Aipermon GmbH, Munich, Germany) (Jehn et al., 2009a,b). Additionally, participants reported total sports activities using the FQPA (Frey et al., 1999). The intensity in this questionnaire is represented in METs based on the updated Ainsworth Compendium (Ainsworth et al., 2011). Based on the available data we calculated the PROCAM Score as previously recommended (Assmann et al., 2002).

Statistical Analysis and Sample Size Calculation

We characterized our cohort by reporting baseline characteristics as mean and standard deviation (SD). Group effects were analyzed by using a one-way ANOVA with a 2-sided 95%-confidence interval or Mann–Whitney-*U*-Test if no normal distribution was assumed. Data distribution was analyzed graphically. Turkey HSD tests were used to differentiate group effects. Linear regression models were used to calculate a potential association between ADmax and AVR, ADmax, and AFarea, as well as to calculate the influence of classical risk factors on arteriolar FID. The graphs were generated in Excel 2016 and RStudio. All statistical tests were performed with RStudio, version 1.1.463 (R Development Core Team, 2008).

To date, no study on PA and retinal endothelial function exists. Therefore, we calculated the sample size based on our previous study where we investigated static retinal vessel diameter in three different groups with a total sample size of 45 participants. AVR differentiated between obese runners, lean amateur and elite runners (Hanssen et al., 2011). Based on an expected slightly higher variability for DVA, we conservatively planned to include 30 participants in each group to detect group differences with ADmax as the main outcome.

RESULTS

Population Characteristics

Thirty-one HA (mean age 60 ± 8 years, 45% female), 33 HS (mean age 59 ± 7 years, 69% female) and 76 SR (mean age 58 ± 6 years, 51% female) were included in the final analysis (**Figure 1B**). Distribution of CV risk factors in SR is shown in **Figure 1A**. Population characteristics are presented in **Tables 1–4**.

Retinal Microvascular Function

Healthy sedentary showed higher FID compared to SR [HS: ADmax = 3.5 (2.1)%; AFarea = 48.2 (31.9)%*s vs. SR: ADmax = 2.7 (1.8)%, $p = 0.021$; AFarea = 34.5 (26.5)%*s, $p = 0.006$] and HA [HA: AFarea = 32.8 (23.1)%*s, $p = 0.029$] (**Figure 2** and **Table 3**). FID in HA and SR did not significantly differ (**Figure 2** and **Table 4**). Median flicker response calculated separately for every second and group is shown in **Figure 3**. We found little evidence for other group differences in ADmax, AFarea, VDmax, and VFarea (**Tables 1–4**). Higher age was significantly associated with reduced ADmax and AFarea. No significant associations were observed for body mass index (BMI), 24 h systolic and diastolic blood pressure, fasting glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride, PA or fitness. However, patients with diabetic medications or elevated fasting glucose levels ($n = 32$) showed reduced ADmax [2.3 (1.7)% vs. 3.2 (1.8)%, $p = 0.092$] and significantly blunted AFarea [24.7 (23.1)%*s vs. 41.7 (26.7)%*s, $p = 0.031$] compared to non-diabetic SR ($n = 44$). Other risk factors were not associated with FID. No gender-specific group differences were observed.

HA showed a higher AVR compared to HS with a further decline in SR (0.87 ± 0.05 vs. 0.83 ± 0.04 vs. 0.79 ± 0.05, $p < 0.001$). Mean AVR in our cohort was 0.82. Of the 84 participants who had an AVR < 0.82, 39 had ADmax values <2.5% and 43 individuals >2.5%. Of the 58 participants who had an AVR > 0.82, 28 had ADmax values <2.5% and 30 individuals >2.5% (**Figure 4**).

Linear regression model between ADmax and AVR showed no statistically significant association [$r(138) = 0.013$, $p = 0.093$]. ADmax and AFarea were highly correlated [$r(138) = 39.35$; $p < 0.001$].

DISCUSSION

Arteriolar FID can differentiate between HS and at risk (SR) individuals with better retinal endothelial function in healthy individuals. However, several individuals in the HA group seemed to present with impaired FID, which was accompanied by a higher AVR. AVR was higher in HA compared to

TABLE 1 | Overall population characteristics.

	HA (*n* = 31) mean (SD)	HS (*n* = 33) mean (SD)	SR (*n* = 76) mean (SD)	*p*
Population characteristics				
Sex (f/m)	14/17	23/10	39/37	0.137
Age (years)	60 (8)	59 (7)	58 (6)	0.286
Height (cm)	171 (7)	168 (9)	169 (8)	0.390
Weight (kg)	63.9 (5.9)	70.8 (9.9)	94.8 (13.9)	**<0.001**
BMI (kg/m^2)	21.9 (1.6)	24.9 (2.5)	33.2 (4.1)	**<0.001**
WC (cm)	82.0 (6.6)	90.1 (8.9)	111.2 (11.6)	**<0.001**
Fat mass (kg)	12.4 (3.8)	22.8 (5.7)	38.0 (9.7)	**<0.001**
Muscle mass (kg)	28.5 (4.2)	26.2 (4.7)	31.6 (7.0)	**<0.001**
Rest systolic BP (mmHg)	127 (16)	128 (15)	132 (14)	0.165
Rest diastolic BP (mmHg)	77 (8)	81 (8)	88 (9)	**<0.001**
24 h. systolic BP (mmHg)	120 (7)	121 (7)	130 (11)	**<0.001**
24 h. diastolic BP (mmHg)	76 (5)	76 (6)	81 (8)	**<0.001**
Fasting glucose (mmol/l)	4.7 (0.4)	4.7 (0.5)	5.8 (1.9)	**<0.001**
Triglyceride (mmol/l)	1.0 (0.3)	1.1 (0.3)	1.8 (1.1)	**<0.001**
HDL (mmol/l)	1.9 (0.4)	1.7 (0.4)	1.3 (0.3)	**<0.001**
LDL (mmol/l)	2.8 (0.8)	3.2 (0.8)	3.1 (0.8)	0.183
PROCAM Score	28.2 (6.5)	32.6 (9.6)	41.3 (9.3)	**<0.001**
Activity and fitness				
FQPA (METS)	44.7 (33.3)	1.9 (2.3)	1.0 (2.1)	**<0.001**
Steps per day (n)	12492 (4230)	10298 (3914)	8697 (3591)	**<0.001**
Walking per day (min)	142 (49)	124 (45)	105 (43)	**<0.001**
VO2max (ml O_2/min)	43.3 (8.7)	29.8 (4.2)	26.1 (4.2)	**<0.001**
Retinal microcirculation				
AVR	0.87 (0.05)	0.83 (0.04)	0.79 (0.05)	**<0.001**
ADmax (%)	2.7 (1.6)	3.5 (2.1)	2.7 (1.8)	0.099
AFarea (%*s)	32.8 (23.1)	48.2 (31.9)	34.5 (26.5)	**0.037**
ACmax (%)	−1.4 (1.2)	−1.3 (1.2)	−1.3 (1.0)	0.807
VDmax (%)	4.2 (1.8)	4.0 (2.0)	4.0 (2.1)	0.914
VFarea (%*s)	41.0 (21.7)	43.6 (25.7)	43.4 (24.6)	0.876
VCmax (%)	−0.9 (0.7)	−0.8 (0.9)	−0.7 (0.6)	0.266

HA, healthy active; HS, healthy sedentary; SR, sedentary at risk; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FQPA, Freiburg questionnaire of physical activity; METS, metabolic equivalents; VO2max, maximal oxygen uptake; AVR, arteriolar-to-venular diameter ratio; ADmax, maximal arteriolar dilatation; AFarea, integral under arteriolar flicker curve; ACmax, maximal arteriolar constriction; VDmax, maximal venular dilatation; VFarea, integral under venular flicker curve; VCmax, maximal venular constriction; SD, standard deviation; p, level of significance for overall group differences (ANOVA). Bold values are statistically significant p-values ($p < 0.05$).

HS with further deterioration in SR. When analyzing the combination of individual dynamic FID with concomitant static retinal vessel diameters, we identified patients with impaired FID but normal AVR and vice versa. Both dynamic FID and static retinal vessel diameters have previously been shown to be associated with CV risk and incidence CV disease. Our current findings pose a diagnostic challenge and need to be addressed in order to put into perspective the use of retinal microvascular function as a diagnostic tool for CV risk stratification.

To date, few data are available on DVA as a new method to assess retinal microvascular endothelial function in health and disease. To verify our results, we need to compare our findings in active and sedentary individuals with previous reports in individuals with low and high CV risk. In our study, sedentary healthy individuals showed an ADmax of 3.5%. In comparison, FID in healthy older individuals, measured by the same flicker protocol, has been previously described to be between 3.6% (Nagele et al., 2018b) and 3.8% (Seshadri et al., 2016), which is in line with our findings. In our study, HS were explicitly screened for sedentary behavior which is likely to explain the slightly lower arteriolar FID compared to previous reports. Sedentary patients with increased CV risk (SR) had a mean ADmax of 2.7% in our study. This is comparable to the few previous reports in patients with CV risk ranging between 2.3% (Nagele et al., 2018b) and 2.4% (Al-Fiadh et al., 2015). The slight difference to previous reports may be explained by a lower CV risk profile in our patients. It can therefore be concluded that our findings of FID in sedentary healthy and diseased individuals stand in good agreement with the available but scarce literature. No study to date has investigated the impact of PA and fitness on retinal endothelial function. Most interestingly, we found a blunted FID (2.7%) in HA which was comparable to our findings in

TABLE 2 | Group differences between healthy active and healthy sedentary individuals.

	HA (n = 31) mean (SD)	HS (n = 33) mean (SD)	p
Population characteristics			
Sex (f/m)	14/17	23/10	0.088
Age (years)	60 (8)	59 (7)	0.799
Height (cm)	170 (7)	168 (9)	0.204
Weight (kg)	63.9 (5.9)	70.8 (9.9)	**0.010**
BMI (kg/m^2)	21.9 (1.6)	24.9 (2.5)	**<0.001**
WC (cm)	82.0 (6.6)	90.1 (8.9)	**<0.001**
Fat mass (kg)	12.4 (3.8)	22.8 (5.7)	**<0.001**
Muscle mass (kg)	28.5 (4.2)	26.2 (4.7)	0.076
Rest systolic BP (mmHg)	127 (16)	128 (15)	0.882
Rest diastolic BP (mmHg)	77 (8)	81 (8)	**0.046**
24 h. systolic BP (mmHg)	120 (7)	121 (7)	0.363
24 h. diastolic BP (mmHg)	76 (5)	76 (6)	0.914
Fasting glucose (mmol/l)	4.7 (0.4)	4.7 (0.5)	0.680
Triglyceride (mmol/l)	1.0 (0.3)	1.1 (0.3)	0.086
HDL (mmol/l)	1.9 (0.4)	1.7 (0.4)	**0.027**
LDL (mmol/l)	2.8 (0.8)	3.2 (0.8)	0.111
PROCAM Score	28.2 (6.5)	32.6 (9.6)	**0.020**
Activity and fitness			
FQPA (METS)	44.7 (33.3)	1.9 (2.3)	**<0.001**
Steps per day (n)	12492 (4230)	10298 (3914)	0.100
Walking per day (min)	142 (49)	124 (45)	0.212
VO2max (ml O_2/min)	43.3 (8.7)	29.8 (4.2)	**<0.001**
Retinal microcirculation			
AVR	0.87 (0.05)	0.83 (0.04)	**<0.001**
ADmax (%)	2.7 (1.6)	3.5 (2.1)	0.152$^{\#}$
AFarea (%*s)	32.8 (23.1)	48.2 (31.9)	**0.029**$^{\#}$
ACmax (%)	−1.4 (1.2)	−1.3 (1.2)	0.611$^{\#}$
VDmax (%)	4.2 (1.8)	4.0 (2.0)	0.639$^{\#}$
VFarea (%*s)	41.0 (21.7)	43.6 (25.7)	0.815$^{\#}$
VCmax (%)	−0.9 (0.7)	−0.8 (0.9)	0.059$^{\#}$

HA, healthy active; HS, healthy sedentary; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FQPA, Freiburg questionnaire of physical activity; METS, metabolic equivalents; VO2max, maximal oxygen uptake; AVR, arteriolar-to-venular diameter ratio; ADmax, maximal arteriolar dilatation; AFarea, integral under arteriolar flicker curve; ACmax, maximal arteriolar constriction; VDmax, maximal venular dilatation; VFarea, integral under venular flicker curve; VCmax, maximal venular constriction; SD, standard deviation; p, level of significance for independent samples t-test or Mann–Whitney-U-Test$^{\#}$. Bold values are statistically significant p-values ($p < 0.05$).

TABLE 3 | Group differences between healthy sedentary and sedentary individuals with increased CV risk.

	HS (n = 33) mean (SD)	SR (n = 76) mean (SD)	p
Population characteristics			
Sex (f/m)	23/10	39/37	**0.037**
Age (years)	59 (7)	58 (6)	0.452
Height (cm)	168 (9)	169 (8)	0.089
Weight (kg)	70.8 (9.9)	94.8 (13.9)	**<0.001**
BMI (kg/m^2)	24.9 (2.5)	33.2 (4.1)	**<0.001**
WC (cm)	90.1 (8.9)	111.2 (11.6)	**<0.001**
Fat mass (kg)	22.8 (5.7)	38.0 (9.7)	**<0.001**
Muscle mass (kg)	26.2 (4.7)	31.6 (7.0)	**<0.001**
Rest systolic BP (mmHg)	128 (15)	132 (14)	0.171
Rest diastolic BP (mmHg)	81 (8)	88 (9)	**0.003**
24 h. systolic BP (mmHg)	121 (7)	130 (11)	**0.002**
24 h. diastolic BP (mmHg)	76 (6)	81 (8)	**0.022**
Fasting glucose (mmol/l)	4.7 (0.5)	5.8 (1.9)	**<0.001**
Triglyceride (mmol/l)	1.1 (0.3)	1.8 (1.1)	**0.042**
HDL (mmol/l)	1.7 (0.4)	1.3 (0.3)	**<0.001**
LDL (mmol/l)	3.2 (0.8)	3.1 (0.8)	0.135
PROCAM Score	32.6(9.6)	41.3 (9.3)	**0.007**
Activity and fitness			
FQPA (METS)	1.9 (2.3)	1.0 (2.1)	0.205
Steps per day (n)	10298 (3914)	8697 (3591)	0.138
Walking per day (min)	124 (45)	105 (43)	0.174
VO2max (ml O_2/min)	29.8 (4.2)	26.1 (4.2)	**<0.001**
Retinal microcirculation			
AVR	0.83 (0.04)	0.79 (0.05)	**<0.001**
ADmax (%)	3.5 (2.1)	2.7 (1.8)	**0.021**$^{\#}$
AFarea (%*s)	48.2 (31.9)	34.5 (26.5)	**0.006**$^{\#}$
ACmax (%)	−1.3 (1.2)	−1.3 (1.0)	0.412$^{\#}$
VDmax (%)	4.0 (2.0)	4.0 (2.1)	0.455$^{\#}$
VFarea (%*s)	43.6 (25.7)	43.4 (24.6)	0.579$^{\#}$
VCmax (%)	−0.8 (0.9)	−0.7 (0.6)	0.820$^{\#}$

HS, healthy sedentary; SR, sedentary at risk; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FQPA, Freiburg questionnaire of physical activity; METS, metabolic equivalents; VO2max, maximal oxygen uptake; AVR, arteriolar-to-venular diameter ratio; ADmax, maximal arteriolar dilatation; AFarea, integral under arteriolar flicker curve; ACmax, maximal arteriolar constriction; VDmax, maximal venular dilatation; VFarea, integral under venular flicker curve; VCmax, maximal venular constriction; SD, standard deviation; p, level of significance for independent samples t-test or Mann–Whitney-U-Test$^{\#}$. Bold values are statistically significant p-values ($p < 0.05$).

sedentary patients at risk (SR; 2.7%). As described before, PA and fitness are associated with reduced CV mortality and improved microvascular endothelial function (Blair et al., 1995; Franzoni et al., 2004; Wen et al., 2011). Why then do HA present with a blunted FID similar to patients with CV risk in the SR group?

A previous conference report from the annual meeting of the Association for Research in Vision and Ophthalmology in 2007 supports our findings of a reduced FID in active individuals. Lovasik et al. (2007) investigated arteriolar FID in ten healthy endurance-trained runners and ten healthy sedentary controls. Runners showed a reduced arteriolar FID (−2.3%) (Lovasik et al., 2007) and wider arteriolar diameters (Kergoat et al., 2008) compared to healthy controls. In our study, HA significantly differed in baseline retinal vessel diameters compared to SR. HA had a higher AVR compared to HS with a further decline in SR. It therefore seems plausible to speculate that the reduced FID in active peers is a sign of a physiologic adaptation to exercise training, leading to arteriolar predilatation and a subsequent reduced dilatation capacity rather than a sign of manifest endothelial dysfunction. The physiologic importance of baseline diameter or dilatation status for interpretation of retinal arteriolar endothelial function has been addressed previously. Neumann et al. (2016) measured retinal vessel diameters as well as FID under normal and hypoxic conditions (Neumann et al., 2016).

TABLE 4 | Group differences between healthy active and sedentary individuals with increased CV risk.

	HA (n = 31) mean (SD)	SR (n = 76) mean (SD)	p
Anthropometry data			
Sex (f/m)	14/17	39/37	0.572
Age (years)	60 (8)	58 (6)	0.315
Height (cm)	171 (7)	169 (8)	0.627
Weight (kg)	63.9 (5.9)	94.8 (13.9)	**<0.001**
BMI (kg/m^2)	21.9 (1.6)	33.2 (4.1)	**<0.001**
WC (cm)	82.0 (6.6)	111.2 (11.6)	**<0.001**
Fat mass (kg)	12.4 (3.8)	38.0 (9.7)	**<0.001**
Muscle mass (kg)	28.5 (4.2)	31.6 (7.0)	**0.001**
Rest systolic BP (mmHg)	127 (16)	132 (14)	0.234
Rest diastolic BP (mmHg)	77 (8)	88 (9)	**<0.001**
24 h. systolic BP (mmHg)	120 (7)	130 (11)	**0.001**
24 h. diastolic BP (mmHg)	76 (5)	81 (8)	**0.018**
Fasting glucose (mmol/l)	4.7 (0.4)	5.8 (1.9)	**<0.001**
Triglyceride (mmol/l)	1.0 (0.3)	1.8 (1.1)	**0.004**
HDL (mmol/l)	1.9 (0.4)	1.3 (0.3)	**<0.001**
LDL (mmol/l)	2.8 (0.8)	3.1 (0.8)	0.762
PROCAM Score	28.2 (6.5)	41.3 (9.3)	**<0.001**
Activity and fitness			
FQPA (METS)	44.7 (33.3)	1.0 (2.1)	**<0.001**
Steps per day (n)	12492 (4230)	8697 (3591)	**<0.001**
Walking per day (min)	142 (49)	105 (43)	**0.003**
VO2max (ml O_2/min)	43.3 (8.7)	26.1 (4.2)	**<0.001**
Retinal microcirculation			
AVR	0.87 (0.05)	0.79 (0.05)	**<0.001**
ADmax (%)	2.7 (1.6)	2.7 (1.8)	0.318$^{\#}$
AFarea (%*s)	32.8 (23.1)	34.5 (26.5)	0.264$^{\#}$
ACmax (%)	−1.4 (1.2)	−1.3 (1.0)	0.205$^{\#}$
VDmax (%)	4.2 (1.8)	4.0 (2.1)	0.247$^{\#}$
VFarea (%*s)	41.0 (21.7)	43.4 (24.6)	0.898$^{\#}$
VCmax (%)	−0.9 (0.7)	−0.7 (0.6)	0.235$^{\#}$

HA, healthy active; SR, sedentary at risk; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FQPA, Freiburg questionnaire of physical activity; METS, metabolic equivalents; VO2max, maximal oxygen uptake; AVR, arteriolar-to-venular diameter ratio; ADmax, maximal arteriolar dilatation; AFarea, integral under arteriolar flicker curve; ACmax, maximal arteriolar constriction; VDmax, maximal venular dilatation; VFarea, integral under venular flicker curve; VCmax, maximal venular constriction; SD, standard deviation; p, level of significance for independent samples t-test or Mann–Whitney-U-Test$^{\#}$. Bold values are statistically significant p-values ($p < 0.05$).

Under hypoxic conditions, retinal arterioles dilated as a physiologic autoregulatory response to low oxygen partial pressure. It was shown that FID was blunted after hypoxia-induced predilatation of the arteriole (Neumann et al., 2016). It therefore seems plausible that exercise-induced dilatation of arterioles may lead to a reduced dilatation reserve and blunted retinal FID.

The following discussion of large artery endothelial function in athletes aims to support and generalize our hypothesis. It is well established that endurance training is associated with peripheral conduit artery remodeling with larger arteries in the exercised limbs (Schmidt-Trucksass et al., 2000; Huonker et al., 2003; Rowley et al., 2011). The existence of an "athlete's artery" has previously been proposed addressing the paradox why endothelial function is not enhanced in long-term trained athletes (Green et al., 2012). Endothelial function, as measured by flow-mediated dilatation (FMD) in the brachial artery, has been shown to be increased, normal or even decreased in athletes, questioning the long-term effects of exercise on arterial function (Green et al., 2013; Montero et al., 2013). The mechanisms remain unclear but the baseline diameter at rest seems to play a key role. Celermajer et al. (1992) found a strong correlation between resting arterial diameter and FMD, a direct measure of endothelial function in the brachial artery (Celermajer et al., 1992). Narrower arteries had a greater dilatation response and healthy subjects with large baseline arteries showed a blunted FMD. These results were confirmed by Rembold et al. (2003). The correlation between FMD and retinal FID are low to moderate (Pemp et al., 2009; Nagele et al., 2018b), nonetheless the underlying mechanisms may indeed be comparable. Both methods measure shear stress-induced and NO-mediated vascular dilatation (Corretti et al., 2002; Dorner et al., 2003) in response to different stimuli. It therefore seems reasonable to hypothesize that the same physiologic principle of a reduced dilatation reserve in predilatated large and small arteries may account for reduced endothelial response in physically active and fit subjects.

To illustrate the resting diameter, we used AVR and not CRAE because CRAE has a high inter-individual variability depending on the magnification factor and the anatomy and height of the individual. AVR represents the ratio between CRAE and CRVE which neutralizes these inter-individual differences. The use of AVR may help put into perspective the role of the arteriolar baseline diameter and the interpretation of FID as a vascular biomarker for CV risk. In **Figure 4** we plotted arteriolar FID against the corresponding AVR for all individuals in our study. Individuals with a high AVR and a coinciding high FID present with a favorable, healthy microvascular phenotype (green area), whereas it appears eminent that persons with a low AVR and a coinciding low FID present with an impaired microvascular phenotype associated with an increased CV risk (red area). However, a high fluctuation of arteriolar FID becomes evident in patients with the same AVR. Several subjects with a favorably high AVR present with low arteriolar FID. At the other end of the scale, several subjects with a critically low AVR present with high FID (gray areas). How can this conundrum be explained?

Individuals with a high AVR but blunted FID are predominantly physically active and fit. An exercise-induced predilatation with a reduced dilatation reserve my lead to the reduction in FID. Individuals with low AVR but high FID are predominantly SR. Differences in functional and structural narrowing of the baseline diameters may help explain this phenomenon. Patients with narrow arterioles and low AVR with normal endothelial function are likely to have functional narrowing of the arterioles, for example due to higher blood pressure. Increase in blood pressure stimulates myogenic vasoconstriction (Bayliss effect) and is associated with functional narrowing of arterioles (Lip and Hall, 2007), which may still

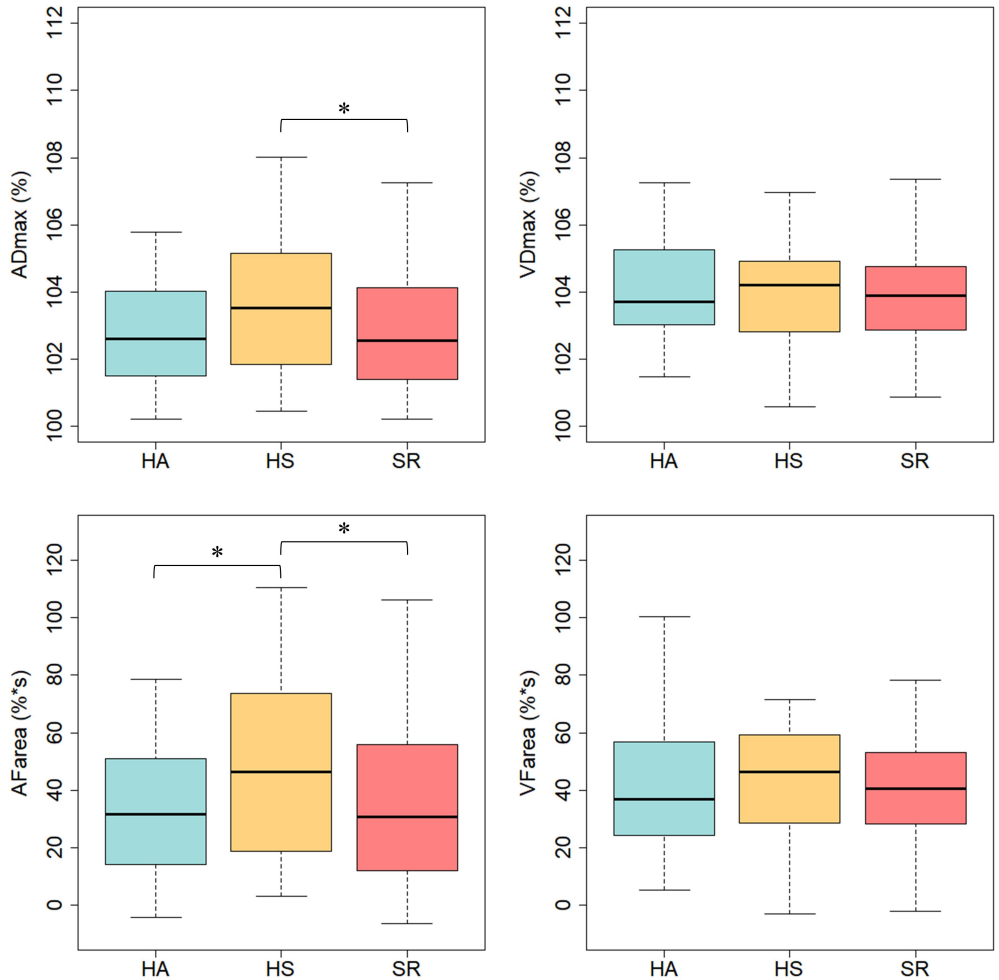

FIGURE 2 | Arteriolar and venular flicker response in healthy active (HA), healthy sedentary (HS) and sedentary individuals with increased CV risk (SR). ADmax, maximal arteriolar dilatation; VDmax, maximal venular dilatation; AFarea, integral under arteriolar flicker curve; VFarea, integral under venular flicker curve; $*p < 0.05$ for Mann–Whitney-*U*-Test.

be reversible. Long-term hypertension may induce structural remodeling and severe vascular damage. Patients with narrow arterioles and low AVR as well as impaired endothelial function are prone to have structural damage, which is less likely to be reversible. It is of utmost interest for future studies to investigate whether these patients differ in long-term CV outcome and prognosis. In our study, no associations of DVA with classic CV risk factors were found. This does not appear to be surprising on the basis of the above arguments. Due to the necessary differentiation of microvascular function in active and sedentary individuals the mere association of risk factors with FID may get blurred. The combined use of static and DVA gives information beyond association of risk factors.

From a clinical perspective it is necessary to define cut-off values for both the arteriolar flicker response and retinal vessel diameters. No such cut-off values have been defined as yet. However, ADmax values between 2.3 and 2.4% or lower have been associated with CV risk factors (Al-Fiadh et al., 2015; Nagele et al., 2018b), diabetes (Sörensen et al., 2016) or heart failure (Nagele et al., 2018b). Lower AVR values are associated with hypertension (Ikram et al., 2006b), diabetes and inflammation (Wong et al., 2006) as well as coronary heart disease (Wong et al., 2002), stroke (Ikram et al., 2006a) and a higher CV mortality (Wang et al., 2007) and AVR levels below the mean of our cohort (0.82) are generally considered as pathological. We therefore set our intra-cohort study cut-off levels at a FID of 2.5% and AVR of 0.82. Definite cut off values need to be defined in future prospective long-term outcome trials. Moreover, there seems to be an urgent need for individual differentiation of the physiologic or pathophysiologic principles underlying retinal microvascular impairments. In an individualized diagnostic approach, a healthy active individual should not be diagnosed with retinal endothelial dysfunction in the presence of a high AVR and in the absence of CV risk factors. In sedentary patients with

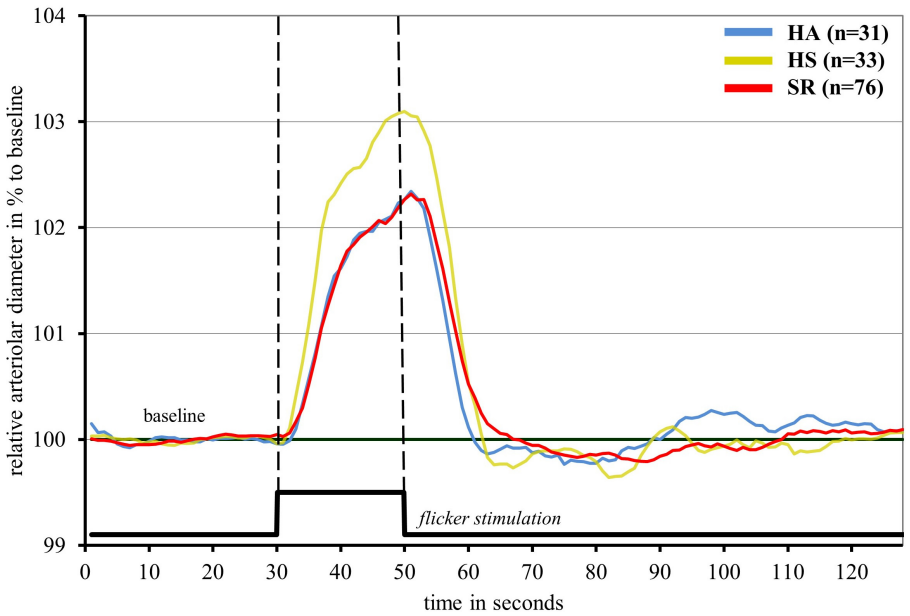

FIGURE 3 | Median arteriolar flicker response in healthy active (blue), healthy sedentary (yellow) and sedentary individuals with increased CV risk (red).

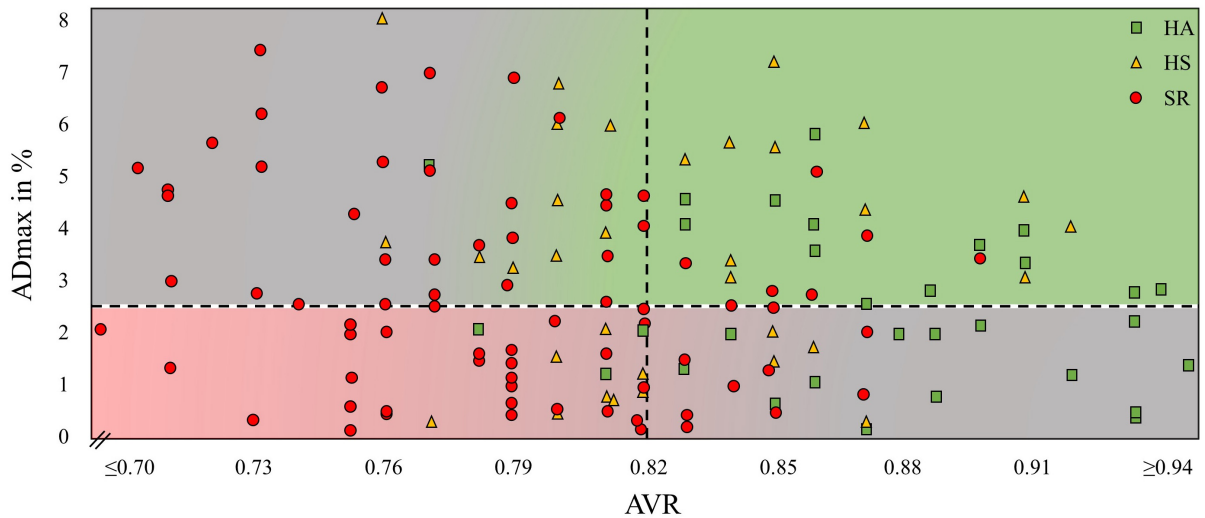

FIGURE 4 | Maximal arteriolar dilatation in % (ADmax) and arteriolar-to-venular diameter ratio (AVR) of every study participant. HA, healthy active; HS, healthy sedentary; SR, sedentary patients with increased CV risk.

known CV risk factors, a sustained normal FID is a good sign, however, a low AVR may indicate functional arteriolar narrowing and a remaining CV risk. In a population-based approach with large cohorts these differentiations may be negligible and may be lost in the statistical deviation. Findings of previous population-based large cohort studies on associations of retinal vessel phenotype with CV risk and risk prediction are very valuable. However, it does not necessarily mean that these findings can equivalently be transferred into a personalized medicine approach. The combination of impaired FID and low AVR are indeed associated with increased CV risk. But for individual risk stratification and treatment recommendations, the proposed differentiation of arteriolar FID in relation to the AVR seems clinically indicated and is strongly recommendable.

This study has some limitations. Participants in our study were between 50 and 80 years old. Our findings and interpretation of results cannot be generalized to other age groups. The discussion of the results is based on sound physiologic principles and previous findings. Nonetheless, we are aware that the

interpretation of our results remains hypothesis-driven. The discussed physiologic mechanisms need to be confirmed in future studies which was beyond the scope of our current study approach. Future research needs to apply patient-orientated differentiated diagnostics on the retinal microvascular phenotype in long-term follow up studies to correctly stratify individual risk and estimate prognosis as well as offer appropriate treatment recommendations. Our study is cross-sectional and effects of therapeutic interventions on retinal microvascular phenotype need to be elucidated. Inclusion criteria for SR were ≥2 CV risk factors out of seven. The study was not designed to discriminate between these CV risk factors. Further research in larger population-based cohorts is needed to evaluate the influence of these CV risk factors on the retinal microvascular function separately.

To conclude, arteriolar FID assessed by DVA differentiates between low and high CV risk in older adults. Physically fit individuals show a blunted FID comparable to patients with CV disease. A possible explanation may be a reduced dilatation reserve as a result of arteriolar predilatation in exercise-trained subjects. Our results demonstrate that a differentiated assessment of retinal endothelial function in combination with retinal vessel diameters is warranted to meet the diagnostic challenge of an individualized personal medicine approach.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Northwest and Central Switzerland.

AUTHOR CONTRIBUTIONS

LS drafted the manuscript, conducted the eye examinations, was responsible for general data collection, and analyzed retinal endothelial function and anthropometric measurements. KK revised the manuscript and discussed the methodological approach. AD conducted the medical examinations, and critically revised the manuscript. DI gave statistical support and revised the manuscript. WV critically discussed the results and revised the manuscript. HH as the principal investigator designed the study, critically discussed the results, and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of Interest Statement: WV is CEO of Imedos Systems.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Publication 5: Association of physical activity and cardiovascular risk with retinal microvascular phenotype: $p66^{Shc}$ expression as a putative mechanistic link

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Abstract

Background: Narrower retinal arterioles and wider venules are linked to worse cardiovascular (CV) outcome. The adaptor protein $p66^{Shc}$ is a key driver of oxidative stress and is modulated by DNA methylation of its promoter. We aimed to investigate the link between physical activity, retinal vessel diameters and the $p66^{Shc}$ pathway.

Design/Methods: Out of 158 subjects (mean age 59.4±7.0 years) included in the cross-sectional study, 38 were healthy active (HA), 36 healthy sedentary (HS) and 84 sedentary at increased CV risk (SR). Central retinal arteriolar (CRAE) and venular (CRVE) diameters and the arteriolar-to-venular diameter ratio (AVR) were measured using a retinal vessel analyzer. Plasma 3-nitrotyrosine (3-NT) was measured by ELISA as a marker of oxidative stress. Gene expression of $p66^{Shc}$ and DNA methylation analysis were assessed in peripheral blood mononuclear cells (PBMCs) by RT-qPCR and Methylminer® qPCR.

Results: Our results demonstrated wider CRAE (179±14µm) and narrower CRVE (204±17µm) in HA compared to HS (CRAE: 172±11 µm; CRVE: 209±11 µm) resulting in a higher AVR in HA (0.88±0.05) compared to HS (0.83± 0.04, $p<0.001$). By contrast, SR showed narrower CRAE (171±14 µm) and wider CRVE (218±16µm, $p<0.05$; AVR: 0.79±0.05, $p<0.001$) compared to HS. In both sedentary groups, higher $p66^{Shc}$ expression and increased plasma levels of 3-NT were associated with hypomethylation of $p66^{Shc}$ promoter.

Conclusions: We conclude that physical activity has the potential to counteract microvascular dysfunction in older subjects. Downregulation of $p66^{Shc}$ expression via DNA methylation may represent a putative mechanistic link whereby active lifestyle promotes healthy microvascular ageing.

Introduction

Retinal vessels represent a window to assess subclinical vascular remodelling and microvascular aging[1]. Retinal vessel analysis as a diagnostic tool can improve CV risk stratification and alterations of retinal vessels have been shown to be predictive of long-term CV outcome[2, 3]. Narrower retinal arterioles and wider venules have been associated with increased CV events in the elderly such as stroke[4], coronary heart disease[5] as well as higher CV mortality[6]. In a previous study, we have found that moderate continuous exercise training can improve retinal microvascular phenotype by modulating the nitric oxide (NO) pathway in lean and obese middle-aged subjects[7]. Whether long-term physical activity can preserve retinal microvascular phenotype in healthy older individuals compared to sedentary healthy and diseased individuals has not been investigated to date.

Endothelial dysfunction is caused by a disturbed homeostasis between NO bioavailability and reactive oxygen species (ROS)[8]. The mitochondrial adaptor $p66^{Shc}$ plays a key role in ageing-induced oxidative stress[9]. We recently reported a significant upregulation of $p66^{Shc}$ in peripheral blood mononuclear cells isolated from patients with type 2 diabetes[10]. This elevated expression of $p66^{Shc}$ correlated with *in vivo* markers of oxidative stress and endothelial dysfunction[10]. Epigenetic signatures at the gene promoter were found responsible for increased $p66^{Shc}$ gene expression[10].

The precise molecular and epigenetic mechanisms of how PA may affect CV health remain to be fully understood. Few studies have shown that regular exercise can modulate methylation levels, which translate into differential gene expression[11], even at the genome-wide level in healthy men and women[12]. The role of PA in epigenetic regulation of $p66^{Shc}$, ROS generation and microvascular phenotype remains unknown.

Methods

Study design

Based on data from the EXAMIN AGE study[13], we investigated the association of long-term PA on retinal microvascular phenotype and analysed DNA methylation of $p66^{Shc}$ promoter as well as systemic oxidative stress levels in healthy older active (HA), healthy older sedentary (HS) and older sedentary with increased CV risk (SR). Inclusion and exclusion criteria are described in detail in the supplement material.

The study was performed at the Department of Sport, Exercise and Health in Basel, Switzerland. The molecular and epigenetic analyses were performed at the Department of Medicine, Karolinska University Hospital, Sweden. The study was planned and conducted in accordance to the protocol and principles stated in the Helsinki Declaration (World Medical Association, 2001). The study approval was obtained from the Ethics Committee of Northwest and Central Switzerland (EKNZ 2015-351). All participants signed a written informed consent. A detailed study protocol has been published[13] and the study has been registered at ClinicalTrials.gov (NCT02796976).

Static Retinal Vessel Analysis (SVA)

The Retinal Vessel Analyzer (RVA, IMEDOS Systems, Jena, Germany) and a fundus camera (450 FF, Carl Zeiss, Jena, Germany) were used to measure retinal vessel diameters after pupil dilatation with Tropicamide 0.5%. Three valid images were taken from one eye at an angle of 50°. The detailed procedure has been described previously[7, 13]. Average diameters were taken as central retinal arteriolar (CRAE) and central retinal venular (CRVE) equivalents using the Paar-Hubbard formula[14]. Retinal vessel diameters are presented in µm, as one measuring unit of the imaging device relates to 1µm in the model of Gullstrand's normal eye. The ratio between CRAE and CRVE was calculated and presented as the arteriolar-to-venular diameter ratio (AVR). To avoid inter-observer variation, all fundus images were taken and analysed by the same experienced investigator who was blinded for group allocations. In our study, we repeated analysis of 30 images and calculated the correlation coefficient (CC) and the coefficient of variation (CVar). The CC for CRAE was r =0.98, the CVar was 8.30%. For CRVE, the CC was r =0.97 and the CVar was 6.27% (AVR: r =0.97 and CVar =9.84%), indicating high reproducibility for all three retinal parameters (P<0.001 each).

Isolation of mononuclear cells and measurement of plasma 3-nitrotyrosine

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll®-Paque (GE Healthcare Europe GmbH, Switzerland) and Leucosep™ 50ml tubes (Greiner BIO-ONE, Germany). Isolated PBMCs were washed in PBS and stored in RLT buffer (RNeasy Mini Kit, Qiagen, Switzerland) at -80°C for DNA and RNA isolation. Plasma was also stored at -80°C. Oxidative stress marker 3-nitrotyrosine (3-NT) was measured in plasma using OxySelect™ Nitrotyrosine ELISA kit (Cell Biolabs, CA, USA) following the manufacturer`s instructions. Plasma

nitrotyrosine levels were determined by comparison with a standard curve prepared from predetermined nitrated bovine serum albumin standards.

RT qPCR

Gene expression of $p66^{Shc}$ was assessed in 20 HA, 20 HS as well as in 40 SR individuals. Gene expression analysis of $p66^{Shc}$ was performed in PBMCs by real-time quantitative polymerase chain reaction (RT-qPCR). RNA was extracted with Direct-Zol™ RNA miniprep kit (Zymo research, CA, USA) and cDNA was synthesized with high capacity cDNA conversion kit (Applied Biosystems, Foster City, CA, USA). mRNA levels of $p66^{Shc}$ gene were detected by RT-qPCR using ABI 7900HT system (Applied Biosystems, Foster City, CA, USA) and FastStart Universal SYBR Green technology (Roche, Basel, Switzerland). Actin-Beta (*ACTB*) gene was used as endogenous control for normalizing RNA concentration. Differences in cycle threshold (Ct) values between test gene and endogenous control (ACTB; ΔCt) were calculated and used for statistical analysis.

Promoter DNA methylation

DNA methylation analysis of $p66^{Shc}$ promoter was performed in the same 20 HA, 20 HS and in 40 SR individuals. Genomic DNA was isolated from PBMCs using phenol:chloroform:isoamyl alcohol (Sigma Aldrich, St. Louis, USA), nucleospin Gel and PCR cleanup kit (Macherey-Nagel, PA, USA). One µg of purified DNA was used to assess methylation status of $p66^{Shc}$ promoter. Methylated cytosines were captured with MethylMiner Kit (Invitrogen, CA, USA) and the level of methylation was assessed with promoter specific primers, ABI 7900HT RT-qPCR system and fluorescence-based FastStart Universal SYBR Green technology (Roche, Basel, Switzerland). Methylated and non-methylated control duplexes provided by the manufacturer were used as controls for methyl-CpG-binding-domain (MBD) protein capture. The amount of DNA captured by MBD protein was normalized to the input (starting DNA material) of each sample. The primer sets used for detection of $p66^{Shc}$ promoter methylation are indicated in supplementary material online, Table S1.

Anthropometry, physical activity and fitness

All anthropometric measurements were performed according to standard procedures described previously[13]. With the use of the Cortex Metalyzer R 3B metabolic test system

(Cortex Biophysik GmbH, Leipzig, Germany), we measured circulatory and ventilatory parameters including VO_2max during an individualized treadmill ramp protocol as previously described[13]. Study participants wore an Aipermotion 440 accelerometer (Aipermon GmbH, Munich, Germany) on their left hip on six consecutive days. We calculated total steps per day and minutes of walking per day using the AiperView 440 and ActiCoach MPAT2Viewer Software (Aipermon GmbH, Munich, Germany) from the five most active days[15, 16]. The self-reported sport activities were assessed using FQPA[17] in metabolic equivalents (METs) per week based on the Ainsworth Compendium[17, 18].

Statistical analysis and sample size calculation

The primary outcome of the cross-sectional approach was the difference in AVR between HA, HS and SR. Boxplots were used for the visualization of primary and secondary outcomes. We applied analysis of variance to compare the AVR and secondary outcomes between HA, HS and SR. Linear regression model was used to analyse the association of VO_2max with AVR. Statistical program R (version 3.5.0) was used for the generation of graphs and for statistical tests with a 2-sided confidence interval of 95%.

Based on previous studies we assumed AVR values of 0.88, 0.83 and 0.78 for HA, HS and SR with a standard deviation of 0.05[7, 19]. To reach a target power of 90% with a 2-sided significance level of 0.05, we needed 36 participants in each group.

Results

We finally included 158 individuals (38 HA, 36 HS and 84 SR) to analyse the association of long-term PA and fitness with retinal vessel diameters, $p66^{Shc}$ expression and oxidative stress levels (Figure S1). All groups were age-matched and differed in anthropometric data (Table 1). Table S2 shows the distribution of CV risk factors of the SR group.

Table 1. Population characteristics.

	HA (n=38) mean (SD)	HS (n=36) mean (SD)	SR (n=84) mean (SD)	*P*
Patients' characteristics				
Sex (f/m)	17/21	26/10	42/42	0.036
Age (years)	60 (7)	60 (7)	59 (6)	0.570
Height (cm)	171.1 (7.7)	167.5 (8.8)	168.9 (8.0)	0.160
Weight (kg)	64.5 (6.5)	70.2 (9.9)	94.7 (14.0)	<0.001
BMI (kg/m^2)	22.1 (1.7)	24.8 (2.4)	33.2 (4.1)	<0.001
WC (cm)	82.1 (6.6)	89.4 (8.9)	111.4 (11.5)	<0.001
Fat mass (kg)	13.0 (3.8)	22.8 (5.9)	37.9 (9.7)	<0.001
Muscle mass (kg)	28.6 (4.4)	25.9 (4.8)	31.6 (6.9)	<0.001
Rest systolic BP (mmHg)	128 (15)	127 (15)	132 (15)	0.594
Rest diastolic BP (mmHg)	78 (8)	81 (8)	87 (10)	0.007
24h. systolic BP (mmHg)	120 (6)	121 (7)	130 (11)	<0.001
24h. diastolic BP (mmHg)	76 (5)	76 (6)	81 (8)	<0.001
Fasting glucose (mmol/l)	4.7 (0.4)	4.7 (0.5)	5.8 (1.8)	0.014
Triglyceride (mmol/l)	0.9 (0.3)	1.1 (0.3)	1.8 (1.1)	0.002
HDL (mmol/l)	2.0 (0.4)	1.7 (0.4)	1.3 (0.3)	<0.001
LDL (mmol/l)	3.0 (0.7)	3.2 (0.8)	3.2 (0.8)	0.535
Activity and fitness				
FQPA (METs)	46.2 (36.3)	1.7 (2.3)	1.3 (2.8)	<0.001
Steps per day (n)	13267 (4869)	10105 (3828)	8711 (3588)	<0.001
Walking per day (min)	148 (52)	121 (44)	105 (43)	<0.001
VO_2max (ml/min/kg)	42.5 (8.3)	29.9 (4.3)	26.0 (4.3)	<0.001

Abbreviations: HA, healthy active; HS, healthy sedentary; SR, sedentary at risk; BMI, body mass index; WC, waist circumference; BP, blood pressure; HDL, high-density lipoprotein, LDL, low-density lipoprotein; FQPA, Freiburg questionnaire of physical activity; METs, metabolic equivalents; VO_2max, maximal oxygen uptake; SD, standard deviation; *P*, level of significance for overall group differences

Retinal microvascular phenotype

HA individuals showed wider arterioles (179±14µm *vs* 172±11µm) and narrower venules (204±17µm *vs* 209±11µm) compared to HS individuals resulting in a significant higher AVR (0.88±0.05 *vs* 0.83±0.04, *P*<0.001) independent of confounders (Figure 1A; Table 2). CRAE did not differ between HS and SR (171±14µm). Wider venules were found in SR (218±16µm, *P*=0.013) compared to HS resulting in a lower AVR (0.79±0.05, *P*<0.001). This finding was

dependent of BMI, blood pressure, CV medications and VO_2max (Table 2). VO_2max (β =0.004, *P*<0.001) adjusted for age and sex explained 27% of AVR variance including the whole study population (R^2=0.268) (Figure S2).

Gene expression of p66^{Shc} and oxidative stress

Gene expression of mitochondrial adaptor p66^{Shc} was significantly elevated in PBMCs isolated from HS and SR as compared to HA at baseline (6.4±5.6 and 6.4±7.7 vs 1.9±0.9 arbitrary units (AU) respectively, *P*<0.01, Figure 1B). In accordance with the upregulation of p66^{Shc}, 3-NT levels were higher in both HS and SR as compared to HA (5.6±3.4μg/ml and 6.0±4.6μg/ml for HS and SR vs 3.8±1.8μg/ml in HA, respectively, *P*<0.05) (Figure 1B).

Promoter DNA methylation of p66^{Shc} gene

The human p66^{Shc} gene has a CpG island within the proximal promoter. DNA methylation analysis of p66^{Shc} promoter was performed using Methylminer combined with qPCR. Three different sets of primers were used to comprehensively examine the methylation status of the p66^{Shc} promoter (-225/+676bp of the transcription start site (TSS)) (Figure 1C). Interestingly, DNA methylation of p66^{Shc} promoter showed lower levels of methylation in HS and SR as compared to HA (Figure 1C).

Table 2. Adjusted group differences of retinal vessel diameters.

		CRAE (µm)		CRVE (µm)		AVR	
	Model	β (95% CI)	*P*	β (95% CI)	*P*	β (95% CI)	*P*
HA	1	6.69 (-1.16; 14.55)	0.111	-4.41 (-13.15; 4.33)	0.458	0.05 (0.03; 0.08)	<0.001
vs.	2	6.02 (-2.79; 14.24)	0.195	-4.32 (-13.47; 4.84)	0.506	0.05 (0.02; 0.07)	<0.001
HS	3	5.41 (-2.56; 13.39)	0.246	-4.58 (-13.76; 4.59)	0.465	0.05 (0.02; 0.07)	<0.001
	4	5.27 (-2.65; 13.20)	0.260	-4.69 (-13.90; 4.49)	0.449	0.05 (0.02; 0.07)	<0.001
	5	9.53 (-0.62; 19.68)	0.070	-1.15 (-13.01;10.66)	0.971	0.05 (0.02; 0.09)	<0.001
HS	1	0.74 (-6.08; 7.56)	0.965	-9.17 (-16.77; -1.58)	0.013	0.04 (0.02; 0.06)	<0.001
vs.	2	-1.27 (-11.11; 8.56)	0.949	-8.89 (-19.85; 2.07)	0.137	0.03 (-0.01; 0.06)	0.120
SR	3	-1.88 (-11.47; 7.72)	0.889	-9.23 (-20.27; 1.80)	0.120	0.02 (-0.01; 0.06)	0.138
	4	-4.47 (-14.66; 5.71)	0.552	-11.14 (-22.90; 0.65)	0.068	0.02 (-0.01; 0.05)	0.292
	5	-3.76 (-13.93; 6.41)	0.656	-10.46 (-22.30; 1.38)	0.095	0.02 (-0.01; 0.06)	0.272
HA	1	7.43 (0.82; 14.04)	0.023	-13.59 (-20.94; -6.23)	<0.001	0.09 (0.07; 0.11)	<0.001
vs.	2	4.75 (-6.76; 16.26)	0.593	-13.20 (-26.04; -0.37)	0.042	0.07 (0.04; 0.11)	<0.001
SR	3	3.54 (-7.70; 14.77)	0.737	-13.81 (-26.74; -0.89)	0.033	0.07 (0.03; 0.11)	<0.001
	4	0.80 (-10.99; 12.58)	0.986	-15.83 (-29.50; -2.18)	0.019	0.06 (0.03; 0.10)	<0.001
	5	5.77 (-13.93; 6.41)	0.656	-11.61 (-27.50; 4.29)	0.198	0.07 (0.03; 0.12)	<0.001

HA, healthy active (n=38); HS, healthy sedentary (n=36); SR, sedentary at risk (n=84); CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; AVR, arteriolar-to-venular diameter ratio; CI, confidence interval; *P*, level of significance for the regression; Model 1, adjusted for age and sex; Model 2, adjusted for age, sex and body mass index (BMI); Model 3, adjusted for age, sex, BMI, systolic and diastolic blood pressure; Model 4 adjusted for age, BMI, systolic and diastolic blood pressure and cardiovascular (CV) medication; Model 5 adjusted for age, BMI, systolic and diastolic blood pressure, CV medication and maximal oxygen uptake.

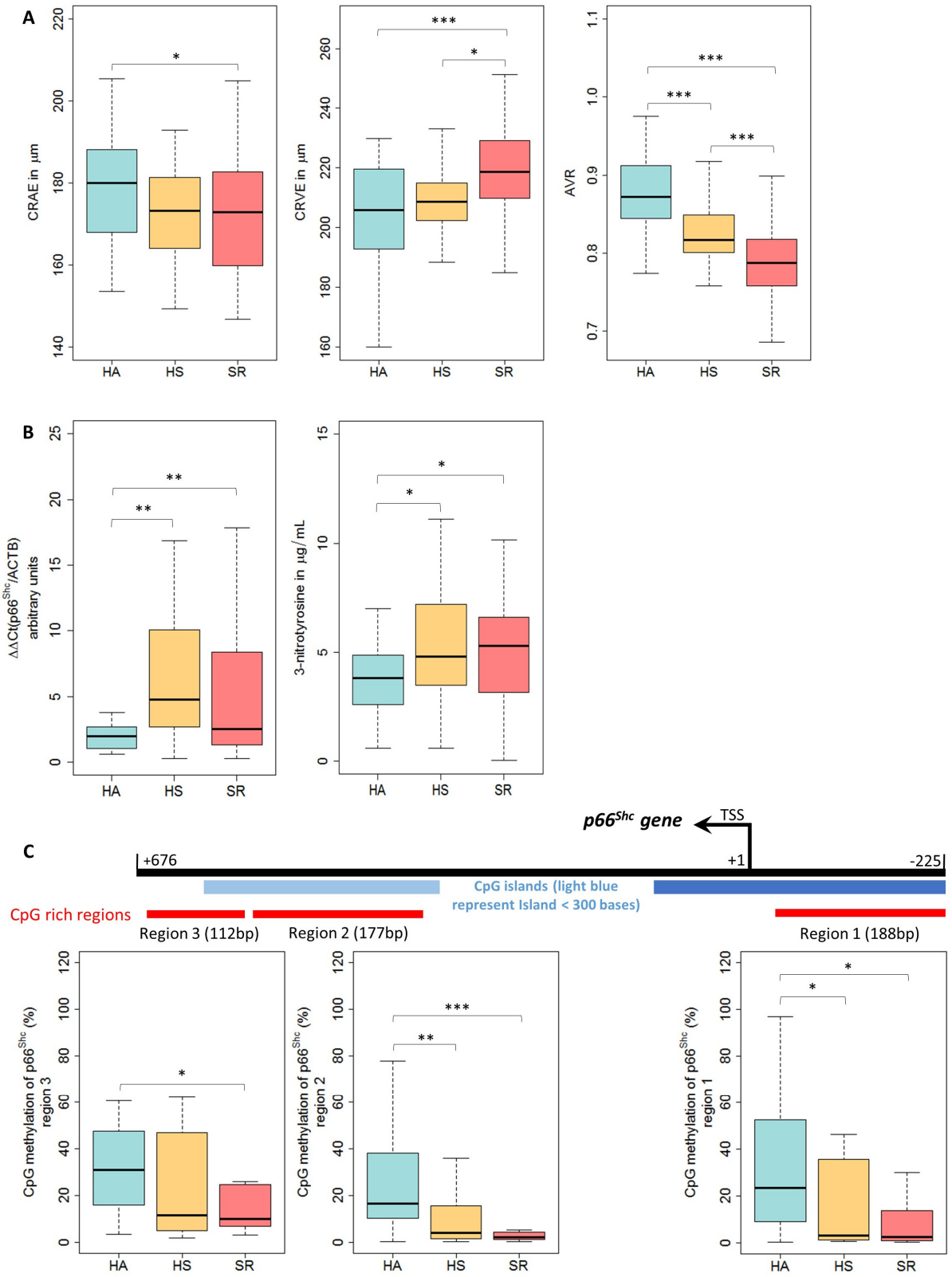

Figure 1. Group specific differences in retinal microvascular phenotype, p66^{Shc} gene expression and 3-nitrotyrosine levels.

(A) Central retinal arteriolar (CRAE) and central retinal venular (CRVE) diameter equivalents as well as arteriolar-to-venular diameter ratio in 38 healthy active (HA), 36 healthy sedentary (HS) as well as in 84 sedentary at risk (SR) individuals. (B) Expression of $p66^{Shc}$ relative to ACTB measured in 20 HA and 20 HS as well as in 40 SR individuals and oxidative stress measured as plasma 3-nitrotyrosine levels in 38 HA, 36 HS as well as in 84 SR individuals. (C) $p66^{Shc}$ gene and CpG islands proximal to $p66^{Shc}$ promoter (red lines indicate CpG rich regions amplified with specific primers). Levels of DNA methylation at region 3, 2 and 1 of $p66^{Shc}$ promoter in 20 HA, 20 HS as well as in 40 SR individuals. Values are expressed as mean±SD. ANOVA *P*-values are shown for multiple comparisons; **P*<0.05, ***P*<0.01, ****P*<0.001. ACTB, actin beta.

Discussion

The present study demonstrates that long-term PA and fitness are associated with a more favourable microvascular phenotype known to be associated with better CV outcome. Moreover, long-term PA in healthy individuals is associated with downregulation of $p66^{Shc}$ gene expression via DNA methylation and a concomitant reduction of oxidative stress levels. Long-term PA was associated with a microvascular phenotype characterized by wider arteriolar and narrower venular diameters. This phenotype has been linked with a lower incidence of coronary heart disease-related deaths[20], atherosclerotic CV events[2], stroke[3] and reduced CV mortality[6]. The fact that 27% of the AVR variance at baseline was explained by cardiorespiratory fitness (VO_2max) emphasizes the importance of improving physical fitness to support healthy microvascular aging. Restoration of NO bioavailability and blunting of oxidative as well as inflammatory processes are likely to play a major role in mediating PA-induced improvements of microvascular function. Our results suggest that healthy microvascular ageing can be achieved by active lifestyle and higher fitness.

HA and HS did not differ significantly with respect to age, sex and CV risk factors but merely differed in long-term PA and fitness levels. Active lifestyle and fitness are therefore considered the most important mediators of lower oxidative stress, lower $p66^{Shc}$ expression and methylation regulators in the HA group. HS and SR did not significantly differ in methylation status, $p66^{Shc}$ expression and oxidative stress levels. This suggests that sedentariness alone may modulate the described epigenetic pathway comparable to the more common CV risk factors.

The results of our study demonstrate that promoter DNA methylation is essential for $p66^{Shc}$ gene downregulation. PA-mediated methylation of $p66^{Shc}$ promotor, subsequent downregulation of $p66^{Shc}$ gene expression and associated reduction of systemic oxidative stress may prove to be key pathways to ensure healthy microvascular phenotype[8, 9]. By contrast, it is well-known that $ONOO^-$ formation originating from the reaction of NO and O_2^- increases 3-NT levels[21, 22]. Accordingly, plasma levels of 3-NT were lower in HA compared to SR subjects. In the HS and SR group, hypomethylation of $p66^{Shc}$ promoter facilitating $p66^{Shc}$ transcription contributed to oxidative stress.

We have previously demonstrated in a rodent model of diabetes and cell culture that hypomethylation of $p66^{Shc}$ promoter causes gene overexpression, oxidative stress and endothelial dysfunction[23]. Inactivation of $p66^{Shc}$ gene seems to protect against age-induced, ROS-mediated endothelial dysfunction, possibly contributing to the extended life span of $p66^{Shc}$ deficient mice[24]. In addition, we have recently shown that adverse epigenetic changes are responsible for continuous $p66^{Shc}$ upregulation, oxidative stress and endothelial dysfunction in patients with type 2 diabetes[10]. Collectively these studies suggest that $p66^{Shc}$ expression regulated by epigenetic changes of DNA complexes contribute to oxidative stress and endothelial dysfunction. DNA methylation has been reported to play a key role in exercise-induced gene expression in skeletal muscle, leukocytes and adipose tissue in a highly gene specific manner[12, 25-27]. PA-induced methylation of $p66^{Shc}$ promoter in circulating PBMCs may be associated with improvements of microvascular phenotype, although the causative link remains to be established in prospective exercise interventions. Our study however, provides first evidence for PA-induced methylation of $p66^{Shc}$ promotor, subsequent downregulation of $p66^{Shc}$ transcription, decreased systemic ROS generation and improved microvascular health as summarized in the conclusion diagram (Figure 2).

Some limitations of the present study are noteworthy. Our study did not aim to assess the whole epigenetic landscape but instead focused on DNA-methylation und expression of a key gene involved in the process of vascular ageing and oxidative stress generation. The study is cross-sectional and our findings are thus associative in nature. Other sources of oxidative stress exist and may have influenced our results. Nonetheless, we were able to provide a proof of concept that epigenetic regulation of $p66^{Shc}$ gene is linked to ageing associated oxidative stress and microvascular phenotype. Our study applies translational clinical research combining epigenetic pathways with microvascular end organ phenotype.

In conclusion, long-term PA is associated with a healthier microvascular phenotype in older adults. Reprogramming of DNA methylation on p66^{Shc} gene promoter may represent a putative mechanistic link whereby PA and fitness protect against age-related oxidative stress. Our findings offer new insights into clinically relevant target pathways of PA and fitness ranging from microvascular amelioration to epigenetic modulation of oxidative stress in older individuals.

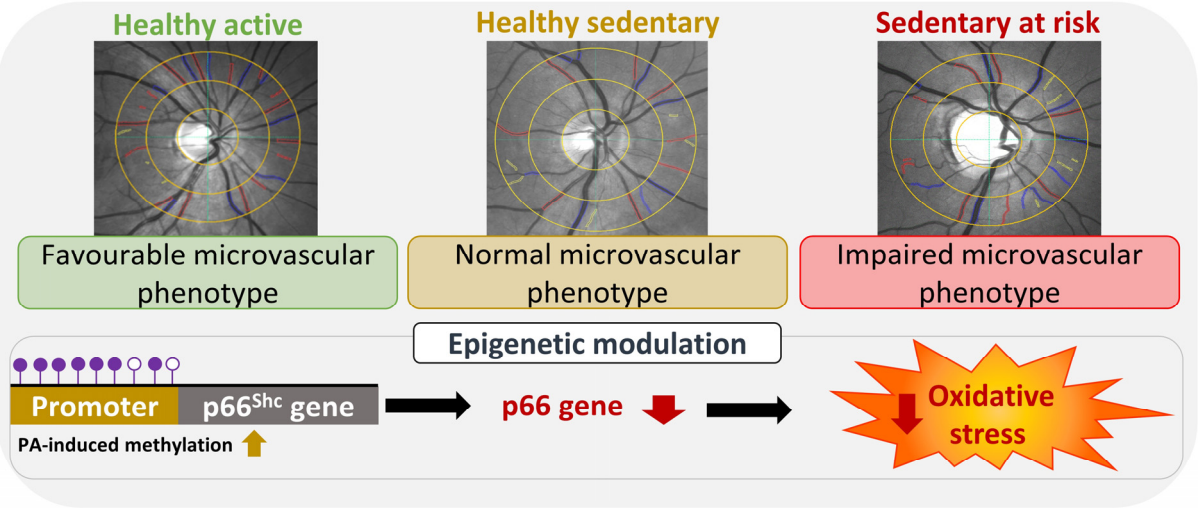

Figure 2. Conclusion diagram.

Healthy active individuals showed a preferential retinal microvascular phenotype compared to healthy sedentary individuals. Sedentary at risk patients showed impaired retinal microvascular phenotype compared to healthy active and healthy sedentary subjects. Preserving the DNA methylation mark on p66^{Shc} gene promoter may represent a putative mechanistic link whereby physical activity protects against age-related oxidative stress in the microcirculation. PA, physical activity.

Author contributions

LS, AWK, FC and HH contributed to the conception or design of the work. LS, AWK, AD, SH, RS, AT, DK, FC and HH contributed to the acquisition, analysis, or interpretation of data for the work. LS and AWK drafted the manuscript. FC and HH critically revised the manuscript. All gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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SUPPLEMENTARY MATERIAL

Methods

Inclusion and exclusion criteria

The recruited men and women were in the age range of 50-80 years. We recruited healthy active individuals (HA) (>9 metabolic equivalents [METs]/week) without any of the CV risk factor described in Table S1 and sedentary individuals (HS) (≤3METs/week) with BMI ≤29.9kg/m^2, as well as sedentary (≤3METs/week) individuals with increased CV risk (SR) (≥two CV risk factors). Exclusion criteria for healthy individuals were history of CVD, pulmonary or chronic inflammatory disease, any of the risk factors described in Table S1, macular degeneration, glaucoma or any chronic eye disease. Exclusion criteria for individuals with increased CV risk were decompensated cardiopulmonary disease or chronic inflammatory disease, chronic eye disease or compromising orthopaedic problems. Based on PA history, self-reported Freiburg Questionnaire of Physical Activity (FQPA), accelerometer data and maximal oxygen uptake (VO_2max), two sports scientists independently judged the level of PA and decided to either allocate the participants into the active or sedentary group or to exclude the subject.

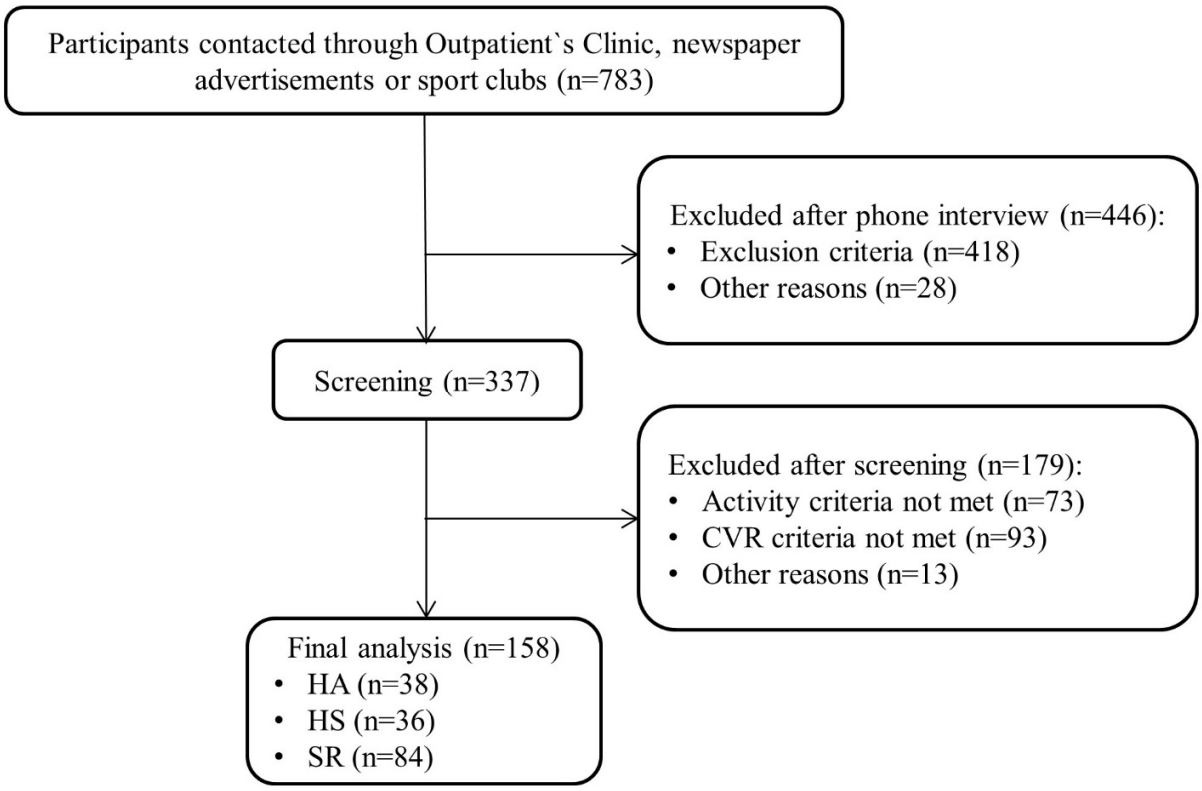

Figure S1. Flow-chart

Abbreviations: HA, healthy active; HS, healthy sedentary; SR, sedentary at risk,

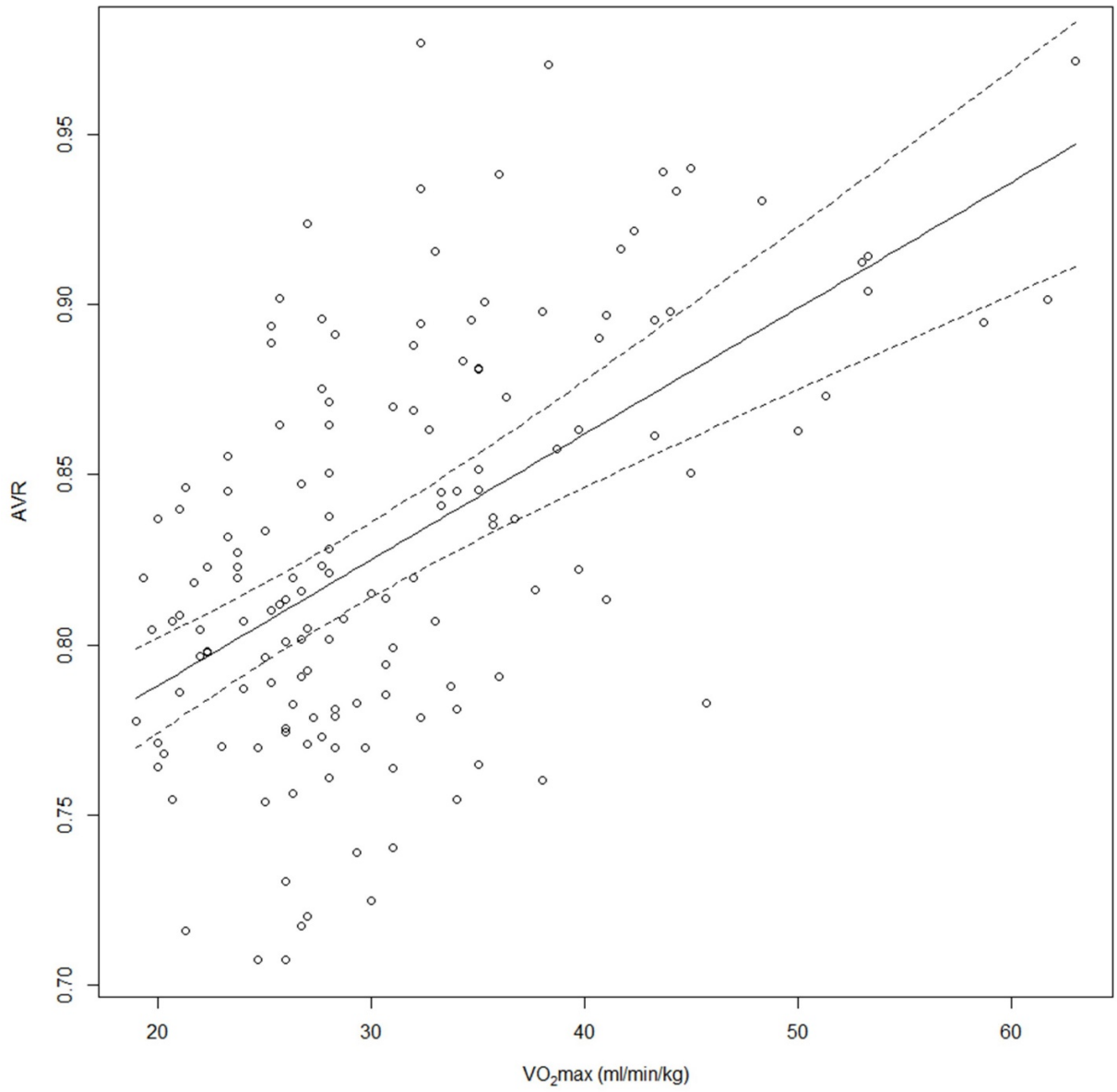

Figure S2. Linear regression model for AVR and VO_2max.

Association of cardiorespiratory fitness and retinal microvascular phenotype in the study population. Abbreviations: AVR, arteriolar-to-venular diameter ratio; VO_2max, maximal oxygen uptake.

Table S1. Primers used in the ExAMIN AGE Study.

Primer Name	Sequence (5'-3')
ACTB_FRT	GTTGTCGACGACGAGCG
ACTB_RRT	GCACAGAGCCTCGCCTT
$p66^{Shc}$_FRT	CTGACACTTTCAAAGCGGTG
$p66^{Shc}$_RRT	GTATGTGCTCACTGGCTTGC
$p66^{Shc}$_CpGisland1_F	TCTACCTCAGGGTCCCTCCT
$p66^{Shc}$_CpGisland1_R	AGCCTCCGATTGGCTTAGAT
$p66^{Shc}$_CpGisland2_F	GGACGCGAACTTCAGACTTC
$p66^{Shc}$_CpGisland2_R	CAACGATCCTCGGCTAACTC
$p66^{Shc}$_CpGisland3_F	GGAGTTTCAGGGATTGACGA
$p66^{Shc}$_CpGisland3_R	GCCCAGAAGTCTGAAGTTCG

Table S2. Risk factors.

risk factor	definition	N (%)
Obesity	BMI ≥30 kg/m^2	71 (85)
Hypertension	≥140 mmHg sys. or ≥90 mmHg dia. BP during 24h monitoring or treatment with antihypertensive medications	18 (21) 38 (45)
Diabetes	fasting glucose ≥5.6 mmol/l or antidiabetic medications	24 (29) 10 (12)
Smoking	current smoking status	28 (33)
Low HDL	<1.0 mmol/l (male); <1.2 mmol/l (female)	28 (33)
Hypertriglyceridemia	triglyceride >1.7 mmol/l	24 (29)
High LDL	>4.9 mmol/l or cholesterol lowering drugs	2 (2) 15 (18)

Abbreviations: BMI, body mass index; sys, systolic; dia, diastolic; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

7. Synthesis, Discussion and Perspectives

This PhD thesis was performed within the EXAMIN AGE study. The first publication "Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population: The EXAMIN AGE Study" is a detailed study protocol of the EXAMIN AGE trial. This chapter summarises and discusses the main results of the thesis, including the accepted manuscript of the second publication "High-intensity interval training modulates retinal microvascular phenotype and DNA methylation of $p66^{Shc}$ gene: a randomized controlled trial (EXAMIN AGE)". In addition, three further manuscripts are summarised and discussed, all of which have been submitted; "Retinal endothelial function in cardiovascular risk patients: a randomized controlled exercise trial", "Retinal endothelial function, physical fitness and cardiovascular risk: a diagnostic challenge" and "Association of physical activity and cardiovascular risk with retinal microvascular phenotype: $p66^{Shc}$ expression as a putative mechanistic link". Taken together, these manuscripts are the backbone of my PhD "Exercise and microvascular health in an ageing population: The EXAMIN AGE study". The three manuscripts will be referred to as publication three, four and five in the following discussion.

7.1 Synthesis

This part of the thesis gives an overview on the main findings of my PhD thesis and addresses the pre-defined hypotheses. Table 1 summarises the main findings of PA and exercise in relation on the retinal microvascular phenotype from all the publications included in my PhD, divided into the cross-sectional and interventional approach. Long-term PA as well as short-term HIIT effects on the retinal vessel phenotype are shown. A favourable retinal microvascular phenotype was previously defined as wider central retinal arteriolar (CRAE) and narrower venular (CRVE) diameters as well as higher flicker light-induced retinal vessel dilatation (FID).

Table 1: Physical activity and exercise in relation to the retinal microvascular phenotype.

		Cross-section (long-term PA)		Intervention (short-term HIIT)	
Method	Retinal phenotype	Group differences	Correlation of phenotype with VO_2peak	Intervention effects	Correlation of Δphenotype with ΔVO_2peak
SVA	CRAE (µm)	HA>SR	n.s.	HIIT>CG	yes
	CRVE (µm)	HA<SR HS<SR	Yes	HIIT<CG	n.s.
	AVR	HA>HS HA>SR HS>SR	Yes	HIIT>CG	yes
DVA	ADmax (%)	HS>SR	n.s.	HIIT>CG	yes
	AFarea (%)	HS>HA HS>SR	n.s.	HIIT>CG	yes
	VDmax (%*s)	n.s.	n.s.	n.s.	n.s.
	VFarea (%*s)	n.s.	n.s.	n.s.	n.s.

Abbreviations: PA, physical activity; HIIT, high-intensity interval training; SVA, static retinal vessel analysis; DVA, dynamic retinal vessel analysis; VO_2peak, maximal oxygen consumption; Δ, delta-change; CRAE, central retinal arteriolar equivalents; CRVE, central retinal venular equivalents; AVR, arteriolar-to-venular diameter ratio; ADmax, maximal arteriolar dilatation; AFarea, arteriolar area under the flicker curve; VDmax, maximal venular dilatation; VFarea, venular area under the flicker curve; HA, healthy active; SR, sedentary at risk; HS, healthy sedentary; n.s., not significant; CG, control group.

7.1.1 Hypothesis 1: Healthy older adults with high levels of PA and fitness have wider retinal arterioles and narrower venules as well as higher FID compared to healthy sedentary peers.

Healthy long-term physically active individuals (HA) showed wider arteriolar (179 ±14µm vs 172 ±11µm, p>0.05) and narrower venular (204 ±17µm vs 209 ±11µm, p>0.05) diameters compared to healthy sedentary individuals (HS) resulting in a significantly higher AVR (0.88 ±0.05 vs 0.83 ±0.04, p<0.001). This result remained significant after adjustment for age, BMI, systolic and diastolic blood pressure, CV medication and VO_2peak. VO_2peak (β = 0.004,

p<0.001) adjusted for age and sex explained 27% of AVR variance observed in the whole study population (R^2 = 0.268). High fitness levels were associated with higher AVR (publication five). HS showed higher area under the arteriolar flicker curve (AFarea) compared to HA (48.2 ±31.9%*s vs. 32.8 ±23.1%*s, p=0.029) (publication four). Our results match the hypothesis that healthy older adults with high levels of PA and fitness show favourable microvascular diameters. However, we did not expect HS to show higher FID compared to HA.

7.1.2 Hypothesis 2: Sedentary older adults with increased CV risk have narrower retinal arterioles and wider venules as well as reduced FID compared to healthy sedentary peers.

Older adults with increased CV risk (SR) showed comparable retinal arteriolar (171 ±14µm vs. 172 ±11µm, p>0.05) but wider venular diameters (218 ±16µm vs. 209 ±11µm, p=0.013) compared to HS resulting in a significantly reduced AVR (0.79 ±0.05 vs 0.83 ±0.04, p<0.001). This result remained significant after adjustment for age and sex but was not independent of group differences in BMI, systolic and diastolic blood pressure, CV medication and VO_2peak (publication five). HS showed higher maximal arteriolar dilatation (ADmax) and AFarea (ADmax = 3.5 ±2.1%; AFarea = 48.2 ±31.9%*s) compared to SR (ADmax = 2.7 ±1.8%, p=0.021; AFarea = 34.5 ±26.5%*s, p=0.006) (publication four). These results are in agreement with our hypothesis that sedentary older adults with increased CV risk show an impaired retinal microvascular phenotype compared to healthy sedentary peers.

7.1.3 Hypothesis 3: Twelve-weeks of HIIT increases central retinal arteriolar diameters, decreases central retinal venular diameters and improves FID in older adults with increased CV risk compared to a control group with standard physical activity recommendations.

After twelve-weeks of HIIT, retinal arteriolar diameters increased (pre: 175 ±14µm vs post: 181 ±13µm, p=0.001) and venular diameters decreased (pre: 222 ±14µm vs post: 220 ±14µm, p=0.007) in SR compared to the control group, while there were no changes observed either for arteriolar (pre: 168 ±14µm vs post: 170 ±16µm, p=0.108) or venular (pre: 214 ±17µm vs

post: 214 ±17µm, p=0.255) diameters. Arteriolar widening and venular narrowing were dependent on changes in VO_2peak. The increased AVR in the intervention group (pre: 0.79 ±0.04 vs post: 0.82 ±0.05, p<0.001) was independent of age, change (Δ) in BMI, systolic and diastolic blood pressure, CV medication and ΔVO_2peak. Exercise-induced arteriolar widening but not venular narrowing remained significant after an intention-to-treat analysis (publication two). Additionally, the HIIT group showed increased ADmax (pre: 2.7 ±2.1%, post: 3.0 ±2.2%, p=0.018) and AFarea (pre: 32.6 ±28.4%*s, post: 37.7 ±30.6%*s, p=0.016) compared to the control group after the intervention (ADmax, pre: 3.2 ±1.8%, post: 2.9 ±1.8%, p=0.254; AFarea, pre: 41.6 ±28.5%*s, post: 37.8 ±27.0%*s, p=0.186). Improvements of ADmax and AFarea remained significant even after adjustment for baseline, age, sex, ΔBMI and mean arterial pressure but were dependent on ΔVO_2peak. There was a significant association between ΔVO_2peak and ΔADmax and ΔAFarea (R^2=0.073, p=0.026; R^2=0.081, p=0.019, respectively). No changes in venular flicker response were observed (publication three). Our findings confirm the assumption that a twelve-week HIIT improves the retinal microvascular phenotype in older adults with increased CV risk.

7.2 Discussion

7.2.1 Exercise as important lifestyle factor to improve retinal microvascular phenotype

Long-term physically active individuals showed wider arteriolar and narrower venular diameters compared to their sedentary peers. This resulted in a significant higher AVR in the active group. This constellation was previously associated with a lower incidence of stroke[99] and coronary heart disease[100] as well as lower CV mortality[101]. Additionally, there is evidence that these favourable diameters are associated with lower risk for hypertension[102, 103], diabetes, obesity, dyslipidaemia and inflammation[100, 104]. It is important to mention that the differences between HA and HS were independent of group differences in age, BMI, systolic and diastolic blood pressure, CV medication and VO_2peak. This shows that long-term PA leads not only to higher CRF, but also to an improved retinal microvascular phenotype independent

of higher fitness levels. In particular, narrower venular diameters and a higher AVR were significantly associated with higher VO_2peak (Table 1). Braun et al. found similar results. Venular narrowing and higher AVR, but not arteriolar diameters, were independently associated with VO_2peak in 260 mainly obese individuals[117]. No correlation between VO_2peak and arteriolar diameters was found[117]. However, increased CRF seems to have a strong impact on retinal microvascular improvements. Looking at the study population as a whole, 27% of the AVR variance at baseline could be explained by VO_2peak, highlighting the importance of high levels of physical fitness for microvascular health. Higher CRF levels were previously associated with lower mortality risk[11, 12, 16, 19, 22] possibly in large part due to improved vascular function compared to relatively unfit individuals (Figure 1). The possible mechanisms responsible for exercise-induced amelioration of the retinal microvasculature will be discussed in chapter "7.2.2 Mechanisms of exercise-induced improvements of retinal microvascular phenotype".

Healthy sedentary individuals with a low CV risk profile (HS) showed comparable arteriolar diameters but significantly narrower venular diameters compared to sedentary individuals with a high CV risk profile, resulting in a reduced AVR in the high CV risk group (SR). These findings were independent of age and sex but dependent on group differences in BMI, systolic and diastolic blood pressure, CV medication and VO_2peak (publication five). In particular, venular widening seemed to explain the AVR differences between these two groups. Wider retinal venular diameters were previously associated with a higher risk of obesity, independent of other risk factors such as hypertension, diabetes or lipids[118]. Sixty-two individuals of SR (74%) were obese, which seems to be a possible explanation for venular widening in this group. In a population-based cohort study of >5 000 individuals of 55 years or older, venular widening was linked to higher inflammation independent of further CV risk factors[119, 120]. Klein et al. showed that venular widening was associated with high serum levels of high-sensitivity C-reactive protein and interleukin 6 after adjusting for age, smoking and diabetes status, serum high-density lipoprotein cholesterol, and haematocrit. The authors concluded that venular widening may be a marker for systemic inflammation[121]. Even SR had a higher fat mass and waist circumference compared to HS (publication five), no significant group differences in mean high sensitivity C-reactive protein levels, a marker of systemic inflammation, were observed between HS (2.0 ±2.9mg/l) and SR (3.6 ±4.1mg/l, p=0.062). High

sensitivity C-reactive protein is an acute-phase and a nonspecific inflammatory protein. More studies with a specific focus on inflammatory markers in relation to retinal vessel diameters are needed to investigate the impact of inflammation on retinal vessel alterations in detail. The multi-ethnic study of atherosclerosis (MESA) demonstrated that arteriolar narrowing and venular widening were both related to higher blood pressure and hypertension, alcohol consumption, higher BMI, diabetes and smoking habits[122]. Interestingly, only venular widening was associated with inflammation[122]. Available literature as well as our findings seem to suggest that the underlying mechanisms for arteriolar narrowing and venular widening are multifactorial as discussed below ("7.2.2 Mechanisms of exercise-induced improvements of retinal microvascular phenotype").

SR showed lower FID compared to HS. FID of HS in our study was comparable to other reports of healthy non-risk cohorts[106, 109] (publication four). SR showed a slightly higher arteriolar dilatation response compared to previous reports of populations at CV risk[106, 123]. The slightly higher arteriolar FID in our cohort can be explained by a lower CV risk profile compared to previous publications using the same flicker protocol (publication four). However, alterations in retinal vessel diameters as well as reduced retinal endothelial function in SR compared to HS lead to the assumption that SR are characterized by an impaired microvascular phenotype, indicative of higher CV risk, advanced vascular ageing and potentially a higher CV mortality (Figure 1)[97, 98, 111].

Patients of the SR group were not previously exercising and had a sedentary lifestyle (publication one) resulting in low CRF levels (VO_2 peak 26.4±3.8 ml/min/kg and 7.5 METs) and, as previously discussed, an impaired retinal microvascular phenotype. We motivated these previously sedentary patients with increased CV risk to participate in a twelve-week HIIT programme. Personal interaction with each participant in a small group setting, the gradual increase of exercise intensity over the first two weeks, supervised training sessions and time allocated for individualised recommendations induced a high degree of motivation and compliance, a positive group dynamic and eventually to a low dropout rate. No adverse effects were observed supporting previous reports of a well-tolerated exercise regime with a low risk for exercise-related CV events during HIIT interventions[46] (publication two and three). SR showed wider arteriolar and narrower venular diameters as well as increased retinal arteriolar

endothelial function (FID) after the twelve-week HIIT. These results demonstrated for the first time that exercise interventions lead to an improved retinal microvascular phenotype in previously sedentary older adults with increased CV risk. Interestingly, post-exercise AVR in SR was restored to levels comparable to HS independent of age, ΔBMI, systolic and diastolic blood pressure, CV medication and ΔVO_2peak, showing that microvascular impairments can be reversed by HIIT independent of classical risk factor improvements in older adults with increased CV risk (publication two and five). In addition, SR improved their CRF from pre VO_2peak of 26.4±3.8 ml/min/kg and 7.5 METs to post VO_2peak of 28.7±4.0 ml/min/kg and 8.2 METs. This corresponds to an improvement in CRF of about 9%. Myers et al. previously demonstrated that each MET increase leads to a mortality risk reduction of about twelve percent[19]. Laukkanen et al. showed a 17-29% risk reduction of non-fatal, and a 28-51% risk reduction of fatal cardiac events per MET increase[20]. As a hypothesis-driven summary it may be concluded that exercise in patients at increased CV risk improves the microvascular phenotype, therefore postponing the process of vascular ageing and potentially reducing CV mortality (Figure 1). If improvement of the retinal microvascular phenotype is really associated with reduction of CV mortality, remains to be elucidated in future follow-up studies.

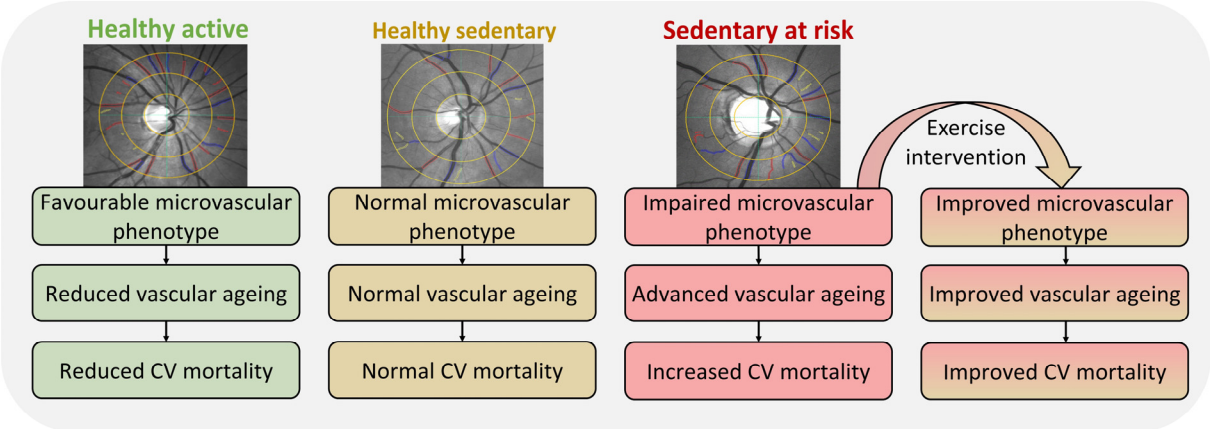

Figure 1. Physical activity and exercise intervention: effects on retinal microvascular phenotype and potential implications for CV mortality.

An active lifestyle leads to favourable retinal microvascular phenotype, which is considered a biomarker for reduced vascular ageing and reduced CV mortality. A healthy but sedentary lifestyle was associated with a normal retinal microvascular phenotype, which is linked to normal vascular ageing and a “normal” CV mortality. A sedentary lifestyle combined with CV

normal vascular ageing and a “normal” CV mortality. A sedentary lifestyle combined with CV risk factors leads to an impaired retinal microvascular phenotype, advanced vascular ageing as well as increased CV mortality. Exercise interventions can improve the retinal microvascular phenotype, which leads to improved vascular ageing and consequently to improved CV mortality.

7.2.2 Mechanisms of exercise-induced improvements of retinal microvascular phenotype

Long-term PA as well as short-term exercise interventions seem to have a beneficial effect on the retinal microcirculation. This chapter will discuss potential mechanisms underlying the microvascular adaptations to exercise training. A key regulator of microvascular function is NO bioavailability as NO is responsible for smooth muscle cell relaxation and vasodilation. Exercise increases blood flow resulting in an increase in sheer stress which stimulates the release of NO[124]. Hanssen et al. demonstrated previously that a ten-week endurance-training programme improved retinal microvascular diameters in healthy lean and obese individuals with different fitness levels. These improvements seemed to be mediated in large part by a higher NO bioavailability[96]. Dorner et al. showed in their experimental approach that FID was significantly reduced after inhibition of NO synthase, supporting the assumption that NO is a key player in the regulation of retinal microvascular function[125]. Paneni et al. described the imbalance between NO bioavailability and ROS as the key driver for vascular dysfunction[59]. Lower ROS levels have been associated with reduced production of O_2^- leading to a higher NO bioavailability[60]. Francia et al. demonstrated in a mouse model that inactivation of $p66^{Shc}$ reduced aortic O_2^- production, which reduced systemic oxidative stress and increased NO bioavailability. These processes seemed to protect against age-related endothelial dysfunction in long-living $p66^{Shc-/-}$ mice[60]. Costantino et al. showed an upregulation of $p66^{Shc}$ gene, oxidative stress and endothelial dysfunction in patients with type 2 diabetes[126]. Endothelial function, $p66^{Shc}$ gene expression and oxidative stress did not change after intensive glycaemic control in these patients[126]. In publication two, we demonstrated for the first time an exercise-induced downregulation of the $p66^{Shc}$ gene via DNA hypermethylation of $p66^{Shc}$ promoter, which resulted in reduced systemic oxidative stress levels. This

modulation of the epigenetic pathway was accompanied by an improved retinal microvascular phenotype, which is a biomarker for healthier vascular ageing. Furthermore, we demonstrated in publication five that long-term physically active individuals showed a hypermethylation of $p66^{Shc}$ promoter, which led to reduced $p66^{Shc}$ gene expression and at the end, to lower systemic ROS levels. Additionally, long-term physically active individuals showed an improved retinal microvascular phenotype suggesting that this epigenetic pathway is a putative mechanistic link whereby PA and CRF protect against retinal microvascular dysfunction. Interestingly, the methylation status of $p66^{Shc}$ promoter, $p66^{Shc}$ gene expression as well as systemic ROS levels did not significantly differ between HS and SR. Physical inactivity and a low CRF alone appear to have a similar impact on this epigenetic pathway compared to sedentariness with increased CV risk.

In addition to higher ROS levels, lipoproteins seems to reduce NO activity[127]. In our cohort, we found a significant group difference in high-density lipoprotein as well as reduced low-density lipoprotein levels after HIIT. In particular, high levels of low-density lipoprotein were previously associated with reduced NO-dependent vascular relaxation[127, 128]. These results lead to the assumption that differences in PA and lipoprotein levels in the cross-sectional approach and improvements in CRF and lipoprotein levels in the interventional approach could be responsible for enhanced NO-dependent vascular function. Another potential mechanism for exercise-induced retinal microvascular benefits are reduced inflammatory cytokines in active individuals. Exercise in general is known for its potential to reduce inflammation[129] and a high degree of inflammation has been previously associated with an impaired retinal microvascular phenotype[104, 111]. However, in our cohort we did not find inflammatory differences between the groups (data not shown) or changes in inflammation after HIIT (publication three). All three groups showed low inflammation, which may explain the lack of significant group differences and exercise intervention effects. Future research seems to be necessary to investigate the influence of PA or exercise intervention on various inflammatory markers and their effects on the retinal microcirculation. Additionally, lower visceral adiposity, improved insulin sensitivity or lower blood pressure could also have influenced the retinal microvascular phenotype indirectly. Previous studies have demonstrated that obesity[96, 115], diabetes[95] and hypertension[102, 103, 107, 115] are associated with an impaired retinal microvascular phenotype. However, in our cohort we did not find any

significant associations with these markers on the retinal microvascular phenotype. Group differences in retinal microvascular phenotype in the cross-sectional approach as well as HIIT effects in the interventional approach were independent of these classical risk factors (publication two and five). However, it cannot be ruled out that these classical risk factors affected the retinal microcirculation in our cohort.

Exercise seems to have no impact on venular FID. Neither significant group effects in the cross-sectional part of the study nor significant training effects in the interventional approach were observed (Table 1). Previous studies demonstrated that venular wall sheer stress is much lower in venular vessels compared to arterioles[130]. This leads to the assumption that the arterioles and the capillary system may buffer the exercise-induced increase in sheer stress, resulting in reduced vascular adaptations in the venular system after exercise. Additionally, FID is mainly affected through a NO-dependent relaxation of smooth muscle cells[125]. The fact that only thin layers of smooth muscle cells surround retinal venular vessels supports the concept that venular vessels are part of a more passive microvascular system leading to a lower exercise adaptivity compared to the arteriolar vessels.

To conclude, NO bioavailability seems to be the key mechanism behind exercise-derived benefits on the retinal microcirculation. However, these benefits on the microcirculation are multifactorial. Further multidisciplinary research approaches are needed for a better understanding of retinal microvascular adaptations in response to exercise and the potential molecular mechanisms.

7.2.3 Retinal vessel phenotype as biomarker of CV risk

This chapter will discuss the potential to use retinal vessel phenotype as a biomarker of CV risk based on our results and previous findings.

In the cross-sectional approach we demonstrated that SVA differentiated between HA and HS. Both groups only differed in their activity status in the absence of CV risk factors. Additionally these findings were independent of group differences in classical risk factors (publication five). This underlines the high sensitivity of SVA in detecting microvascular changes, a biomarker of

vascular impairments, at a subclinical stage, independent of classical CV risk factors. This result supports previous findings of Seidelmann and colleagues. They demonstrated that the assessment of SVA, in addition to classical risk factor stratification, leads to a more precise reclassification from low to intermediate risk in every fifth woman with a low CV risk[97]. This demonstrates, on the one hand, the high sensitivity in detecting subclinical microvascular alterations before classical CV risk factors occur, and on the other hand, the high potential to use SVA additionally to classical CV risk assessments to enhance the timely and subclinical diagnosis of advanced microvascular impairments. A timely diagnosis of microvascular alterations has a high potential to reduce morbidity and mortality by initiating therapies at a stage of the disease where its progression is still modifiable. This would also lead to lower morbidity and mortality rates and an extreme reduction in health care costs.

This PhD work has demonstrated for the first time that retinal vessel analysis (SVA and DVA) can be used to monitor treatment efficacy in older adults with increased CV risk. HIIT leads to improved retinal microvascular phenotype in SR (publication two and three). This has high clinical relevance because monitoring treatment efficacy is essential in a personalised medicine approach. Physicians may use these methods to differentiate non-responders and to intensify treatment strategies to achieve better vascular end-organ function. The benefits for patients are likely to be multifold, spanning from rescuing of the microvascular phenotype, better treatment adherence and motivation to the improvement in quality of life and potentially reduction of CV morbidity and mortality.

In publication four, we discussed the diagnostic challenge of retinal endothelial dysfunction using the DVA. DVA differentiated between low-risk (HS) and high-risk (SR) individuals but seemed to be limited especially in highly active individuals because HA and SR showed comparable FID. In both groups, FID was significantly reduced compared to HS. We plotted the DVA and SVA results in one figure (see publication four Figure 3) to better understand the relation between individual FID and retinal vessel diameters. In this figure, we used the AVR and not the CRAE because the diameter equivalents have a high inter-individual variability depending on the magnification factor and the anatomy of each participant. We identified individuals with a high AVR and a high FID, which is associated with a favourable microvascular phenotype, and individuals with a low AVR and a low FID associated with impaired

microvascular phenotype. Interestingly, several patients with increased CV risk showed low AVR but high FID. Differences of functional or structural narrowing of arterioles seems to be responsible for this phenomenon. Patients with narrow arterioles and a low AVR with normal FID are likely to have functional narrowing of the arterioles, for example due to higher blood pressure at the time of measurement. High blood pressure stimulates myogenic vasoconstriction (Bayliss effect) and is associated with functional narrowing of arterioles[131] leading to higher dilatation reserve. This is more likely to be reversible compared to structural vascular remodeling and vascular damage due to long-term hypertension, characterized by a combination of narrow arterioles and low AVR as well as impaired FID. At the other end of the scale, several individuals with high AVR showed low FID. Individuals with high AVR and blunted FID were predominantly healthy and very active individuals. We tried to explain this phenomenon of a blunted FID in HA by pre-dilated arterioles as a physiological adaptation to long-term exercise training (publication four). Exercise training may lead to arteriolar pre-dilated arterioles and a higher AVR as well as a consequent reduced dilatation capacity and cannot be taken as a sign of manifest endothelial dysfunction. In population-based large cohorts studies this phenomenon does not become apparent due to the large sample size and statistical averaging. However, for individual risk stratification and treatment recommendations the proposed differentiation of arteriolar FID in relation to the AVR seems to be strongly recommendable to meet the diagnostic challenge of an individualized personal medicine approach.

Combining our results with the previously described evidence in chapter “1.3.2 Retinal vessel analysis in CV disease”, I would like to conclude that SVA and DVA are both assessments with a high potential to enhance CV risk stratification and monitor treatment efficacy. However, based on our results it seems to be essential to combine both measurements in an individualised approach to reliably detect microvascular dysfunction as well as to reduce false-positive and false-negative diagnoses.

7.3 Strengths and limitations

This project was the first to investigate the long-term PA and short-term exercise effects on the retinal microvascular phenotype in older adults with and without CV risk. However, our results cannot be generalised for other age groups. Although there are some indications that retinal microvascular diameters also improve in adolescents[116] and younger adults[96] after exercise, more studies are needed to confirm our findings. The short-term exercise effects were investigated after a HIIT programme. More studies with other exercise programmes, for example MCT, are needed to find the best exercise modalities to improve retinal microvascular phenotype in different age and patient groups.

In publications two and five we investigated the epigenetic modulation of oxidative stress via $p66^{Shc}$ methylation status and $p66^{Shc}$ gene expression. For the first time we combined exercise as a treatment option and retinal microvascular phenotyping as an innovative new microvascular diagnostic approach with the assessment of potential epigenetic mechanisms. This is a very important and essential step to better understand the mechanistic cascade from epigenetic modulation to microvascular end-organ phenotype. However, the entire mechanistic landscape needs to be investigated in future studies to prove the causality of our results. The systemic impact of single molecular pathways on the microcirculation remains a future scientific challenge.

In the EXAMIN AGE study, we described for the first time the combination of SVA and DVA in healthy active and sedentary patients with and without increased CV risk. Some patients with impaired retinal vessel diameters showed higher FID compared to healthy and very active individuals who showed favourable retinal microvascular diameters but reduced FID. These results seemed contradictory first but may well be explained by physiological principles, such as functional narrowing and pre-dilated arterioles. It seems essential to combine SVA and DVA to differentiate underlying microvascular impairments and their potential causes.

We did not investigate other previously applied retinal biomarkers for CV risk such as branching or tortuosity of the retinal vessels. As a future perspective, we are using the EXAMIN

AGE data to develop new biomarkers for CV risk stratification in the retinal microcirculation, such as retinal vessel wall thickness or retinal pulse wave velocity.

7.4 Conclusions

Based on the previously described evidence on the demographic change, with an increasing number of individuals living longer and getting older, more people will be affected by chronic non-communicable diseases, CV disease being the most common and at the same time most challenging health burden. The timely diagnosis of vascular damage and dysfunction, conceptually termed early or advanced vascular ageing, and the timely initiation of treatment strategies even at older age are main health care challenges to reduce the growing socioeconomic burden of CV disease. The results of this PhD project support the growing evidence that long-term PA and higher CRF are associated with a favourable microvascular phenotype in older adults, which seems to have the potential to postpone vascular ageing and eventually reduced CV mortality. Furthermore, we demonstrated that older adults with increased CV risk showed an impaired but modifiable retinal microvascular phenotype. The impaired microvascular phenotype is reversible by means of exercise therapy, independent of classical risk factor reduction, even in older adults with increased CV risk. These improvements are associated with reduced vascular ageing and reduced CV mortality. Exercise-induced hypermethylation of the $p66^{Shc}$ promoter led to a downregulation of the $p66^{Shc}$ gene expression, which may represent a putative mechanistic link, whereby exercise protects against age-related oxidative stress.

Additionally, our results demonstrated that a combined assessment of retinal endothelial function with retinal vessel diameters is essential to meet the diagnostic challenge of an individualised personal medicine approach. However, retinal vessel analysis seems to have the potential to improve CV risk stratification by screening subclinical microvascular alterations and monitor treatment efficacy. Improvements in CV risk stratification have the potential to optimize CV prevention programmes as well as to reduce future health care costs.

7.5 Outlook

This PhD project demonstrates the clinical and diagnostic importance of SVA and DVA to differential individual CV risk on a microvascular level. It helps to add new physiological and mechanistic insights into the clinical application of the method under consideration of both benefits and possible pitfalls of the diagnostic approach. Moreover, this work demonstrates the beneficial long-term impact of PA and the short-term effects of intensive exercise training on microvascular health in the elderly. The work is suggestive of possible epigenetic involvement in the process of exercise-induced microvascular amelioration. However, several questions remain to be addressed in the future. To date there are various approaches, software use and protocols available to measure retinal vessel diameters and dynamic retinal endothelial function. A standardisation of these methods and protocols would facilitate comparability. Additionally, publication four showed the need for more evidence to differentiate structural from function alterations in the interpretation of combined SVA and DVA results. It seems to be clear that narrower arteriolar and wider venular diameters as well as reduced FID are associated with increased CV risk and mortality. However, clear cut-off values for SVA or DVA do not exist. Furthermore, no therapy guidelines exist as to how patients with reduced FID or retinal vessel diameter alterations should be treated. More representative data on healthy cohorts are warranted to define normal values. It is of great interest for clinicians in their daily routine to have mandatory guidelines of how to measure and interpret SVA and DVA results to enhance CV risk stratification in an individualised personal medicine approach. The retinal microcirculation was previously described as “window to the heart”[112]. While our work supports this notion, it remains to be elucidated whether the exercise-induced improvement in retinal microvascular phenotype can reduce CV outcome in older adults.

If these questions can be addressed in the years to come, retinal vessel analysis may well have the potential for wide-spread use in daily clinical practice. Image analysis needs further simplification for use outside qualified research centres and the combined use of static and dynamic analysis needs to be transferred from a population-based approach to an individual patient-centred differentiated diagnostic approach. We used PA and exercise as a treatment concept in healthy older and diseased individuals and were able to demonstrate the potential

of retinal vessel analysis for optimization of CV risk stratification, treatment monitoring and future treatment guidance. Exploration of the association and effects of molecular and epigenetic pathways on retinal microvascular function may help develop new treatment strategies to combat the growing burden of CV disease and the associated health care costs.

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9. Appendix

9.1 Publication 6: Short- and Long-Term Effects of Bariatric Surgery on Vascular Phenotype

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ORIGINAL CONTRIBUTIONS

Short- and Long-Term Effects of Bariatric Surgery on Vascular Phenotype

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Abstract

Background Retinal microvascular diameters and large artery stiffness are valid biomarkers of cardiovascular risk. This study assessed short- and long-term micro- and macrovascular improvements after bariatric surgery (BS).

Methods Sixteen patients (44 ± 12 years) underwent BS in this observational study. Two weeks before as well as 6 weeks and 4 years after surgery, retinal vessel analysis and assessment of brachial-ankle pulse wave velocity (baPWV), cardio-ankle vascular index (CAVI), and anthropometry were performed. Three patients were lost to follow-up.

Results Six weeks after BS, retinal arteriolar diameters (CRAE) were wider (180.1 μm vs. 188.1 μm; $p = 0.001$), and the arteriolar-to-venular diameter ratio (AVR) was higher (0.82 vs. 0.86; $p < 0.001$) compared to baseline levels. During the 4 years of follow-up, the retinal changes sustained but further improvements did not occur. Both indices of large artery stiffness, baPWV and CAVI, remained unchanged 6 weeks and 4 years after surgery.

Conclusions Retinal microvascular phenotype improved 6 weeks after BS. The improvements in microvascular health were maintained during 4 years of follow-up but, despite significant further reductions in body mass index, did not improve further long-term. baPWV and CAVI were unaffected after surgery indicating that BS primarily affects microvascular phenotype rather than large artery stiffness. Retinal vessel imaging seems to be a feasible diagnostic tool to monitor microvascular health after BS. Normalization of BMI and blood pressure may be necessary to achieve long-term improvement of large artery phenotype after BS.

Keywords Retinal microcirculation · Arterial stiffness · Obesity · Bariatric surgery · Pulse wave velocity · Cardio-ankle vascular index

Non-standard Abbreviations

AVR	retinal arteriolar-to-venular diameter ratio
baPWV	brachial-ankle pulse wave velocity (m/s)
BMI	body mass index (kg/m^2)
BS	bariatric surgery
CAVI	cardio-ankle vascular index
CRAE	central retinal arteriolar equivalent (μm)
CRVE	central retinal venular equivalent (μm)
MAP	mean arterial pressure (mmHg)
PWV	pulse wave velocity (m/s)

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Introduction

Bariatric surgery (BS) reduces cardiovascular morbidity and mortality [1–3] by improving associated risk factors, such as high body fat, inflammatory status, glucose and lipid metabolism, blood pressure, and left ventricular diastolic function [4–6]. Several studies show that these pleiotropic effects of weight-loss by surgery translate into reduced cardiovascular risk by improving different vascular biomarkers. Habib et al. showed a reduced intima-media thickness and higher flow-mediated dilation in 50 subjects 24 months after BS [7]. Aortic elastic properties and left ventricular diastolic function improved during a 36 months follow-up after BS in 60 subjects [8]. Shargorodsky et al. showed improved large arterial elasticity, which is assessed by pulse wave contour analysis in

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21 patients with a high cardiovascular risk 16 weeks after BS [9]. Carotid intima-media thickness was reduced in a type 2 diabetic population 12 months after BS [10]. Bäckdahl et al. found a reduced aortic pulse wave velocity (PWV) in 82 subjects 2 years after BS and associated this effect to a reduction in white adipose tissue [11]. However, the effects of BS on the microvascular structure and function are less clear. Nerla and Tarzia showed an improved microcirculatory coronary blood flow in response to intravenous adenosine application and cold pressure test 3 months and 4 years after BS [12, 13]. Martin-Rodriguez et al. found improved post-occlusive reactive hyperemia in the forearm skin in obese patients without signs of metabolic syndrome after BS [14].

No study to date has investigated the combined short- and long-term effects of BS on macro- and microvascular phenotypes in the same population. It remains to be shown which vascular bed is most sensitive to the metabolic changes after BS. Retinal vascular diameters and arterial stiffness are sensitive micro- and macrovascular biomarkers, which may be used for monitoring cardiovascular risk after BS. Therefore, the aim of this observational study was to assess short- and long-term improvements of both, small and large artery structure and function 6 weeks and 4 years after BS.

Methods

Study Design

Between January and June 2012, we recruited participants scheduled for BS at St. Claraspital Basel (Switzerland). After signing the informed consent, participants had to attend three appointments: the pre-surgery appointment 2 weeks before surgery and two post-surgery appointments 6 weeks and 4 years after surgery. The surgery was performed in the Claraspital Basel. All measurements and data analysis were performed in the Department of Sport, Exercise, and Health of the University Basel in accordance with the Declaration of Helsinki. The local ethical committee (Ethikkommission Nordwest- und Zentralschweiz 2015-00250) approved the study.

Inclusion and Exclusion Criteria

Inclusion criteria were a (body mass index) BMI > 40 kg/m^2 or a BMI > 35 with comorbidities, such as hypertension or diabetes. Exclusion criteria were age > 65 years, macular degeneration, glaucoma, or any chronic eye disease, which might affect the retinal microcirculation.

Assessment of Anthropometric Data

Assessment of anthropometric data included standardized measurement of height, total body mass, waist circumference, and BMI.

Macrovascular Assessment

Patients were advised to refrain from moderate and vigorous physical activity for 24 h, to refrain from smoking on the day of assessment and to remain fasting for 12 h with the exception to drink water or unsweetened tea up to 2 h prior to the appointment. We used the non-invasive oscillometric VaSera VS-1500N vascular screening system (Fukuda Denshi Co. Ltd., Tokyo, Japan) to obtain brachial-ankle pulse wave velocity (baPWV) and cardio-ankle vascular index (CAVI). Both parameters are well established and widely used as surrogate markers of arterial stiffness and are associated with overall cardiovascular morbidity and mortality [15]. Reproducibility of this method is excellent with mean variation coefficients of 3.9% for baPWV and 4.4% for CAVI, respectively [16]. The basic methodological principle has been described elsewhere, and the procedure was conducted as previously described [16]. After the participant had rested for at least 10 min in a lying position, two measurements on each side were taken in a supine position at 3–5-min intervals. Measurements with a good or very good quality, quantified by the VaSera System, were used for further analysis. Additionally, heart rate and oscillometric blood pressure were measured non-invasively twice at each arm with a VaSera VS-1500N device. Systolic and diastolic blood pressure and mean arterial pressure (MAP = [2 × diastolic blood pressure + systolic blood pressure] / 3) were calculated as the mean of two measurements on the left and two on the right side.

Microvascular Assessment

After 5minutes of rest in a sitting position, retinal microvascular imaging was conducted with a static retinal vessel analyzer (SVA-T, Imedos Systems UG, Jena, Germany). For static retinal vessel analysis, we analyzed two valid pictures from both eyes with an angle of 45° and the optic disc in the center. Venular and arteriolar diameters were analyzed semi-automatically by coursing through an area of 0.5–1 disc diameter from the optic disc margin, at higher magnification and using special analyzing software (Vesselmap 2, Visualis, Imedos Systems UG). Diameters were averaged to the central retinal arteriolar equivalent (CRAE) and the central retinal venular equivalent (CRVE) by using the Parr-Hubbard formula [17]. The retinal arteriolar-to-venular ratio (AVR) was calculated from the CRAE and CRVE. The inter-observer and intra-observer interclass correlation coefficient for the measurement of retinal vessel diameters ranges from 0.97 to 0.98.

Statistical Analysis and Sample Size Calculation

Data analysis was performed with R version 3.4.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria). Descriptive analysis included means and standard deviation (SD). The level of significance was set at $p < 0.05$. To analyze the progression of our main outcome (AVR) from baseline to 4-years follow up, we used multi-level modeling as an analysis tool for repeated measures data [18]. Model selection was based on Akaike's information criterion. In case of significance, we applied post hoc tests for pairwise analysis. In the presence of repeated measurements, observations are no longer independent, which makes usual analysis methods, such as linear regression model unsuitable for the analysis of our data. Multilevel models (or linear mixed models) are regression models that take the correlation between repeated measurements into account [18].

Based on previous findings, we assumed an expected difference in AVR between baseline and 4-years follow-up of 0.04 with a SD of 0.08 [19]. A total sample size of 15 patients was needed to reach a target power of 80% with a two-sided significance level of 0.05. G*Power software 3.1.9.2 was used for the sample size calculation.

Results

Patients' Characteristics at Baseline and 6 Weeks and 4 Years After Bariatric Surgery

Sixteen participants with a mean age of 44 ± 12 years (22–61 years, 13 male and three female) at the baseline appointment were included in this trial. Three participants refused to participate in the follow-up appointment after a mean follow-up of 4 years (Fig. 1). Three patients were smokers and seven were ex-smokers. Fasting metabolic blood parameters at baseline were total cholesterol (5.1 ± 1.1 mmol/L), high-density lipoprotein (1.4 ± 0.3 mmol/L), low-density lipoprotein (3.1 ± 0.9 mmol/L), triglycerides (1.4 ± 0.6 mmol/L), fasting blood glucose (5.2 ± 1.1 mmol/L), and HbA1c (5.7 ± 0.4%). The inflammatory markers were high sensitivity C-reactive protein (5.0 ± 4.6 mg/L) and leucocytes (7.4 ± 2.0 × 10^9/L). Markers for hepatic function were aspartate aminotransferase (25.2 ± 6.7 U/L), alanine aminotransferase (33.8 ± 18.3 U/L), and γ-glutamyltransferase (42.3 ± 17.8 U/L). Ten participants took anti-hypertensive medication, and ten patients were on lipid-lowering medication. The surgical method of choice was laparoscopic sleeve gastrectomy in five patients and Roux-en-Y gastric bypass in 11 patients. Anthropometric measures and blood pressure improved throughout the study period (Table 1). Three participants reduced their anti-hypertensive medications after 6 weeks post-surgery, and seven participants reduced their anti-hypertensive medications until the follow-up after 4 years. Despite improved blood pressure, three patients still had high-normal values, and six patients were hypertensive 4 years after BS. The mean weight loss during the study period was 39.5 kg (Fig. 2). Despite extensive weight loss, six patients were still overweight and obesity remained in seven patients after 4 years.

Microvascular Changes after Bariatric Surgery

The retinal microcirculation showed wider arteriolar diameters 6 weeks and 4 years after BS compared to baseline, without further significant improvements between 6 weeks and 4 years after BS (Table 1). Venular diameters did not change between the time points. AVR increased 6 weeks and 4 years after BS compared to baseline, without further changes between 6 weeks and 4 years after BS. Microvascular short- and long-term effects were independent of MAP changes ($p < 0.01$).

Macrovascular Changes After Bariatric Surgery

CAVI and baPWV did not change significantly during the follow-up period compared to baseline in the models without and with adjustment for MAP (Table 1).

Discussion

This study was designed to investigate microvascular and macrovascular improvements 6 weeks and 4 years after BS. Our preliminary results demonstrated an improved microvascular phenotype independent of blood pressure changes in the early post-surgical phase after 6 weeks. CAVI and baPWV did not change, suggesting that the macrovascular system might be less sensitive to early metabolic changes after BS than the microvascular system. Neither retinal vessel diameters nor CAVI or baPWV further improved during the 4 years of follow-up despite ongoing body weight reduction in our patients.

Short- and Long-Term Changes in Microvascular Function After BS

Subacute improvements in the coronary microcirculation and peripheral endothelial function after BS have been shown before [12]. Our results demonstrate that retinal vessels might also be sensitive to the surgery-related metabolic and physiological changes in the early post-surgical period. These changes are reflected by reductions in blood pressure and weight loss, which might therefore be the main contributors for early microvascular improvements after BS. Blood pressure changes are known as an important factor for alterations of retinal arteriolar diameters

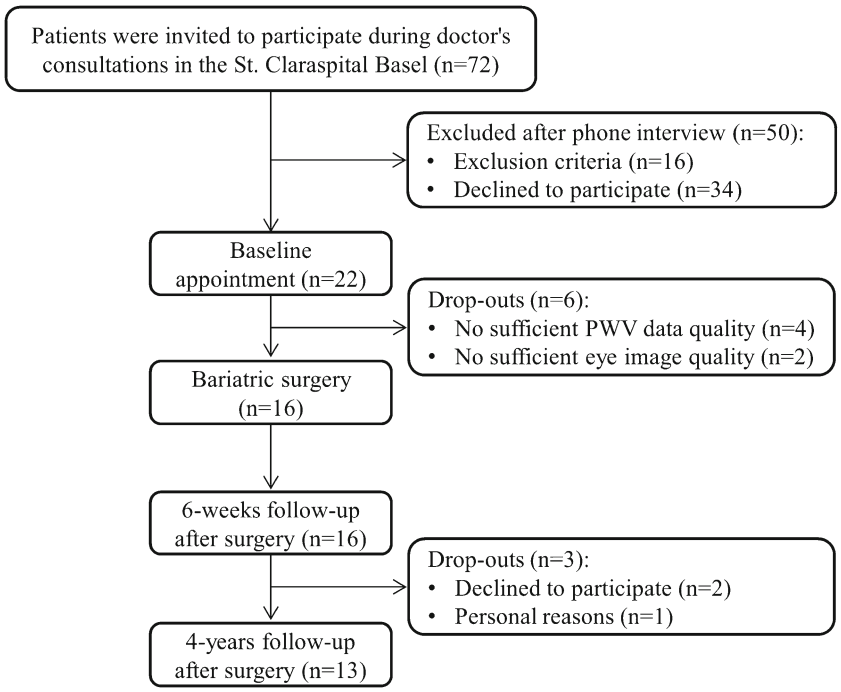

Fig. 1 Flow-chart. PWV = pulse wave velocity

[20]. Nevertheless, we demonstrated an MAP-independent increase in AVR based on wider arteriolar diameters, indicating that there are blood pressure independent mechanisms responsible for the microvascular improvements. A previous meta-analysis demonstrated that higher BMI is associated with narrower arterioles and wider venules [21]. Systemic inflammation has been argued to be responsible for wider venular diameters in patients with obesity [21]. Nevertheless, CRVE did not change in our study population after BS, despite considerable improvements in body composition. As overweight or obesity were still prevalent in most patients at this point,

Table 1 Anthropometric and vascular parameters at baseline ($n = 16$), 6 weeks (6w-post; $n = 16$), and 4 years (4y-post; $n = 13$) after BS

	Baseline	6w-post		4y-post		
	Mean ± SD	Mean ± SD	p^a	Mean ± SD	p^b	p^c
Anthropometry						
Weight (kg)	124.4 ± 19.4	111.9 ± 17.4	< .0001	88.2 ± 16.0	< .0001	< .0001
BMI (kg/m^2)	43.0 ± 4.4	39.1 ± 3.5	< .0001	30.4 ± 3.1	< .0001	< .0001
WC (cm)	125.7 ± 15.3	118.4 ± 13.8	0.0492	95.2 ± 15.3	< .0001	< .0001
Sys. BP (mmHg)	138.9 ± 14.0	129.4 ± 10.3	0.0041	134.5 ± 22.3	0.8866	0.0030
Dia. BP (mmHg)	83.8 ± 8.1	80.4 ± 5.1	0.0801	82.0 ± 10.7	0.9924	0.0977
MAP (mmHg)	104.0 ± 7.4	96.7 ± 6.0	0.0126	99.5 ± 14.1	0.9547	0.0127
Vascular parameter						
CAVI	6.6 ± 1.6	6.8 ± 1.2	0.6895	6.8 ± 0.9	0.9943	0.7849
baPWV (m/s)	12.0 ± 1.9	12.0 ± 1.3	0.5667	12.0 ± 1.8	0.9158	0.3881
CRAE (μm)	180.1 ± 17.0	188.1 ± 17.5	0.0011	186.1 ± 18.4	0.7938	0.0005
CRVE (μm)	218.0 ± 16.6	219.5 ± 16.4	0.4885	215.0 ± 14.8	0.5915	0.9973
AVR	0.82 ± 0.06	0.86 ± 0.05	0.0002	0.87 ± 0.07	0.1110	< .0001

BMI = body mass index, WC = waist circumference, Sys. BP = systolic blood pressure, Dia. BP = diastolic blood pressure, MAP = mean arterial pressure, CAVI = cardio-ankle vascular index, baPWV = brachial-ankle pulse wave velocity, CRAE = central retinal arteriolar equivalent, CRVE = central retinal venular equivalent, AVR = retinal arteriolar-to-venular diameter ratio

[a] p value baseline vs 6w-post

[b] p value 6w-post vs 4y-post

[c] p value baseline vs 4y-post

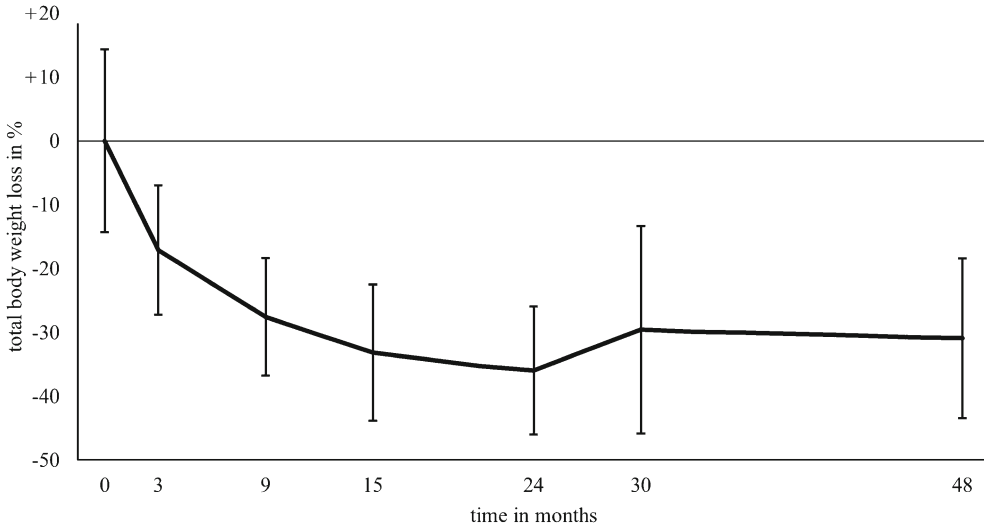

Fig. 2 Mean post-surgical weight loss. Body weight at baseline (0 months) and during follow-up ($n = 13$) after bariatric surgery. Bars depict standard deviation

normalization of body weight might be necessary to achieve relevant improvements of CRVE.

In contrast, retinal arterioles widened in our study population post-surgery. Stapleton and colleagues showed that the bioavailability of nitric oxide is reduced in an obesogenic environment and that weight loss may lead to an attenuation of this deficit [22]. Therefore, increased bioavailability of nitric oxide after BS may be a main reason for the widening of arterioles. Further studies should include biomarkers of systemic inflammation, glucose, and lipid metabolism in order to identify possible metabolic effects of BS on the microcirculation.

Tarzia and colleagues showed no further changes of subacute improvements in micro- and macrovascular function 4 years after BS despite ongoing weight loss and reduced cardiovascular risk factors in 19 patients [13]. However, they used flow mediated dilation and ultrasound-guided coronary flow reserve as markers of vascular function. Thus, our study is the first to investigate transition of short-term to long-term changes in retinal microvascular health compared to large artery stiffness after BS. Our results are in line with findings of Tarzia and colleagues, showing no additional improvement of microvascular health despite ongoing weight loss and reduced waist-circumference. Based on our results and two previous studies on the effects of BS on retinal microvascular health, we would like to postulate the following assumptions: The positive effects of BS on retinal microvascular health seem to occur during the first post-surgical year [19, 23]. We performed a short-term follow-up after 6 weeks in our study, and the effects of BS were more or less immediate. Lammert et al. [18] found wider arterioles, narrower venules, and a higher AVR 9 months after BS. However, the time of post-surgical follow-up varied from six to 23 months in their sample of 30 patients. Bachmayer et al. [22] also observed an improved microvascular phenotype in a population of 21 patients 10 months after BS. They reported narrower venules and a higher AVR, whereas the retinal arterioles were unaffected after BS. Depending on the development of the individual risk profiles of the patients after BS, amelioration of retinal microvascular health is detected by improvements of either retinal arteriolar or venular diameter or both. This finding is supported by the above-mentioned meta-analysis by Boillot et al. [20], which demonstrated that obesity is associated with either retinal arteriolar narrowing or venular widening. In line with our findings, retinal arteriolar diameters have been found to be more strongly associated with the CV risk profile in obesity.

Further improvements in microvascular health after BS do not seem to be facilitated by further continuous weight loss alone. Although considerable weight loss was achieved in our patients, overweight or obesity was still prevalent, and blood pressure levels were still elevated or high-normal in most of our patients. Further improvements and actual amelioration of microvascular health may depend on achieving normal values for weight, blood pressure, and metabolic regulation. In addition to dietary measures and drug treatment of comorbidities, an active lifestyle may play a key role in improvement of microvascular health. We have previously shown that endurance exercise training can reverse alterations of retinal vessel diameters in individuals with obesity [24]. A tendency towards further improvement of AVR after 4 years was detected and may also have reached statistical and clinical significance in a larger cohort.

Short- and Long-Term Changes in Macrovascular Function After BS

In contrast to the microvascular markers, CAVI and baPWV did not change during the early post-surgical phase, despite reductions in blood pressure. These results are in contrast to previous reports, which found a reduction in PWV after weight loss [25, 26]. However, baseline baPWV was not elevated in all but two of our patients, and a post-surgical

improvement was seen only in these two participants. Accordingly, the previously reported independent effect of weight loss on PWV might occur in people with increased PWV but not in those with baPWV values within the normal range [5]. Our observation that CAVI and baPWV did improve neither short-term nor long-term after BS seems to stand in contrast to several other studies [27, 28]. Based on our data, we would like to speculate that baPWV and CAVI do not improve after BS unless pre-surgical values are elevated. Moreover, most of the above referenced studies assessed carotid-femoral PWV, which is calculated including the superficial distance between the two measurement points [29]. Hence, this method is vulnerable to overestimation of PWV especially in individuals with extensive central obesity [30]. Therefore, the use of carotid-femoral PWV for monitoring of changes in arterial stiffness after BS-induced weight loss carries the risk of misinterpretation, as a reduction in follow-up measurements might simply reflect the concurrent weight loss. Utilization of baPWV might be advantageous in studies with participants having morbid obesity, as the simplified measurement of pulse wave travel distance, using linear regression of body height, eliminates body surface-related measurement errors [31]. This is also the reason why we applied measures of systemic (global) arterial stiffness in our study setting. We further considered the oscillometric nature of baPWV and CAVI measurement to enhance accuracy compared to tonometric measurement of arterial stiffness. This study is the first to use markers of systemic arterial stiffness (baPWV and CAVI) rather than central arterial stiffness to assess changes in arterial wall integrity after BS. However, systemic arterial stiffness may not be the best marker to pick up changes in arterial stiffness after BS. Future studies should consider the concomitant use of diagnostic methods to assess both central and systemic arterial stiffness in patients with metabolic disease.

Previous studies showed an association of PWV with collagen depositioning, serum elastase activity, and metalloproteinase-9, suggesting that obesity might cause arterial stiffening by promotion of vascular wall remodeling [32]. The regression of such profound structural alterations is likely to take longer than a few weeks. Hence, post-surgical improvements of central arterial stiffness may need months or years to occur. This suggests that the macrovascular system might be less sensitive to early metabolic changes after BS than the microvascular system. However, in our study, baPWV and CAVI did not change during the 4-year follow-up period despite ongoing weight loss and blood pressure reduction. The normal baseline values of baPWV and CAVI might help explain the results of our study. Moreover, arterial stiffness was our secondary endpoint, and the study was not powered to detect changes in large artery stiffness. Larger sample size may be necessary to detect significant changes in arterial stiffness after BS.

Limitations

This study was powered for the main outcome AVR. Due to the patients lost to follow-up, the long-term follow-up was slightly underpowered. Nevertheless, the retinal microcirculation seems to be sufficiently sensitive to monitor vascular regeneration after BS in a short- and a long-term setting. Future prospective larger scale studies are warranted to investigate the predictive value of retinal microvascular health for long-term cardiovascular outcome after BS.

In patients with metabolic syndrome, the effect of BS on micro- and macrovascular health may be different compared to metabolically healthy patients [14]. Hence, it is important to note that the small sample size of our study did not allow grouping of our patients according to metabolic criteria, such as glucose tolerance, insulin sensitivity, blood lipids, free fatty acids, and inflammatory markers. The normal baseline values of baPWV and CAVI might be responsible for the lack of significant results for these macrovascular biomarkers. In terms of their interpretation, it is important to note that current reference values only exist for central Asian populations [33, 34]. We did not monitor changes in lifestyle behavior. Changes in physical activity and eating habits may influence weight loss and should therefore be considered in future studies on vascular changes after BS. Three patients did not attend the follow-up appointment 4 years after BS. However, tendencies of changes in retinal vessel analysis parameters, CAVI and baPWV in the early post-surgical appointment, were in line with those of the whole study cohort.

Conclusions and Perspectives

This study is the first to investigate early post-surgical changes of macro- and microvascular phenotype in patients with morbid obesity 6 weeks after BS and to track long-term effects over a 4-year follow-up period. CAVI and baPWV were used for the first time to investigate macrovascular effects of BS. We found an amelioration of the microvascular phenotype 6 weeks after BS, which was maintained but not improved any further after 4 years, despite further long-term reduction in BMI. Large artery stiffness remained unaffected after BS both short- as well as long-term. Despite extensive weight loss, normalization of body weight and blood pressure might be necessary to achieve further improvements of vascular health and risk reduction. In the future, a larger prospective cohort study should aim at exploring the underlying mechanisms and causality of post-surgical changes in micro- and macrovascular phenotype. Our results indicate that retinal vessel phenotyping may proof to be a valid diagnostic and more sensitive diagnostic tool than large artery stiffness to monitor development of cardiovascular risk after BS.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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9.2 Publication 7: Exercise and arterial stiffness in the elderly: A combined cross-sectional and randomized controlled trial (EXAMIN AGE)

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Abstract

Objectives: Arterial stiffness (AST) is a main determinant of cardiovascular (CV) mortality. Long-term physical activity (PA) is considered to decrease age-related progression of AST but effects of short-term exercise interventions on AST remain unclear.

Design: In a combined cross-sectional and interventional study approach, we investigated the effects of long-term PA and short-term high-intensity interval training (HIIT) on AST in an older population.

Methods: 147 older individuals (mean age 59±7 years) were assigned to three groups according to their PA and CV risk profile and compared: healthy active (HA, n=35), healthy sedentary (HS, n=33) and sedentary at risk (SR, n=79). In addition, SR were randomized to either 12 weeks of HIIT or standard recommendations. Pulse wave velocity (PWV) was measured by applanation tonometry. Cardiorespiratory fitness (CRF) was performed by symptom-limited spiroergometry to determine maximal oxygen uptake (VO_{2max}).

Results: Higher CRF was associated with lower PWV (p<0.001) and VO_{2max} explained 18% of PWV variance. PWV was higher in SR (8.2±1.4 m/s) compared to HS (7.5±1.6 m/s) and HA (7.0±1.1 m/s; p<0.001). 12 weeks of HIIT did not change PWV in SR. HIIT-induced reduction in systolic BP was associated with reduction in PWV (p<0.05).

Conclusion: SR show higher PWV compared to HS and long-term PA is associated with lower PWV. Reduction of AST following short-term HIIT seems to depend on a concomitant decrease of blood pressure. Our study puts into perspective the effects of long- and short-term exercise on arterial wall integrity as treatment options for CV prevention in an older population.

Introduction

Cardiovascular (CV) diseases are responsible for the majority of deaths in western countries and age has been identified as a main risk factor[1, 2]. Vascular tissue biomarkers such as arterial stiffness (AST) provide a means of optimized risk assessment to detect individual subclinical organ damage. Commonly measured as central pulse wave velocity (PWV), AST has gained clinical importance and has been proven to be a reliable predictor for CV risk in the general population[3]. Its addition to standard care can significantly improve CV risk prediction for the individual with a reclassification rate between 13-15%[3, 4]. Altered PWV indicates subclinical target organ damage and may be used to quantify cumulative damaging effects of CV risk factors on the aging arterial wall integrity.

High cardiorespiratory fitness (CRF) is associated with reduced all-cause and CV mortality[5]. Previous studies on the effect of regular physical activity (PA) and exercise on indices of AST in the elderly have reported conflicting results[6-8]. High-intensity interval training (HIIT) is an exercise modality that has attracted attention for its potency to increase CRF and reduce CV risk in patients, for example, with metabolic syndrom[9].

Data on HIIT and its effects on PWV are scarce. Previous evidence suggests that HIIT may be superior regarding reductions in AST compared to moderate aerobic training in young patients with increased CV risk[10, 11]. However, a recent meta-analysis by Way et al. could not detect differences in AST reduction between the two training regimens [12]. The effects of HIIT on AST in elderly people with increased CV risk have not been investigated to date. Our aim was to investigate the associations between long-term PA and central PWV in healthy and diseased elderly. Moreover, we aimed to examine the effects of 12 weeks of HIIT, defined as short-term exercise, on PWV in diseased elderly with clinical indications for add-on exercise treatment.

Methods

Study design and subjects

The EXAMIN Age study (Exercise, Arterial Crosstalk-Modulation and Inflammation in an Ageing Population) is a combined cross-sectional and interventional study. In the cross-sectional part of the study, elderly participants aged between 50 and 80 years were recruited and assigned to three groups according to their PA and CV risk profile: healthy active (HA), healthy

sedentary (HS) and sedentary individuals with increased CV risk (SR). In the interventional part, SR were randomized into either a walking-based HIIT, performing a supervised training for 12 weeks, or control condition receiving standard PA recommendations only[2]. Participants were recruited by advertisements in local newspapers as well as flyer distribution. Simple randomization was performed by drawing pieces of paper from an envelope by the study physician.

All study visits took place between January 2016 and December 2017. The initial medical screening included a clinical assessment, 24-hour blood pressure (BP) measurement and blood sampling. If participants met inclusion criteria, two additional appointments were arranged to perform vascular measurements and assessment of CRF. Follow-up measurements in SR included three identical appointments after 12 weeks. Each participant provided written informed consent and the study design was approved by the local ethics committee and registered in advance. The study has been reported according to the CONSORT standards[13] and was performed according to the Helsinki Declaration for Good Clinical Practice[14].

Inclusion and exclusion criteria

Inclusion criteria were as follows: Participants were aged between 50-80 years. HA and HS individuals had to be healthy without CV risk factors, whilst SR allocation required at least two of the following CV risk factors: high blood pressure (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic during 24h monitoring or antihypertensive medications), obesity (body mass index ≥ 30 kg/m^2), high fasting plasma glucose levels (≥ 5.6 mmol/l or antidiabetic medications), high triglyceride levels (≥ 1.7 mmol/l), low high-density lipoprotein levels (< 1.0 mmol/l (male); < 1.2 mmol/l (female)), high low-density lipoprotein levels (> 4.9 mmol/l or cholesterol lowering drugs) and current smoker. Additional exclusion criteria for healthy participants were history of CV, pulmonary or chronic inflammatory diseases. Exclusion criteria for individuals at risk were decompensated cardiopulmonary disease and chronic inflammatory diseases as well as compromising orthopaedic problems. PA of each participant was judged combining the participant`s PA history, questionnaire-based self-reported PA, objective accelerometers and maximal aerobic capacity (VO_{2max}). Two sport scientists independently estimated the individual PA level and, if consensus was achieved, participants were assigned to the appropriate group. Further information on PA and CRF evaluation,

anthropometry and blood sampling are presented in the supplementary materials (Supplement S1).

Pulse wave velocity

PWV was measured according to recommendations of current guidelines[15]. To assure standardization, vascular measurements were performed in the morning and participants were asked to refrain from exercise 24 hours and from alcohol and caffeine consumption 12 hours prior to the examination. Participants had to rest 10 minutes in a supine position after systolic and diastolic BP at rest were taken with a cuff from the right brachial artery, using an automatic BP monitor system (Omron Healthcare, Germany). PWV was measured using a standard device by use of applanation tonometry (SphygmoCor CPV®, ATCor Medical, Australia). High quality measurements with a deviation in pulse waveforms of less than 10% within 10-second recordings were considered valid. The mean value of two valid measurements with a mean difference ≤ 1 m/s was used for further calculations. Central PWV in m/s was calculated as distance divided by transit time of carotid and femoral pulse wave (foot-to-foot method). The distance for carotid-femoral PWV was determined by subtracting the suprasternal notch (SSN) to the carotid site distance from the SSN to the femoral site. All analyses were performed by the same experienced investigator who was blinded for group allocation.

Exercise intervention

In the interventional part of the study, a 12 week nordic walking-based HIIT, was applied in the SR group three times per week. Training sessions were supervised by sport scientists. Stepwise increase of intensity in the first two weeks was conducted to familiarize former sedentary participants with aerobic exercise. After two weeks, 4x4 minutes of HIIT at an intensity of 80-90% of maximum heart rate (HR_{max}) was performed. Recovery time between each interval lasted three minutes and was set at an intensity of 60-70% HR_{max}. Including warm-up and cool-down, an average training session lasted 60 minutes.

Statistical analysis

Participants` characteristics are presented with mean (standard deviation (SD)) for continuous variables and frequency counts for categorical variables. Analysis of variance (ANOVA) was used to detect overall group differences in the cross-sectional approach. Multiple linear regression was performed to identify between group differences adjusting for possible confounders. The assumptions of the regressions were verified using residual plots. In the interventional approach we performed an analysis of covariance (ANCOVA) to detect and quantify differences between HIIT and control group adjusted for baseline values[16]. A multiple linear regression model was used to describe factors explaining the difference in central PWV following HIIT. We used two-sided tests with a significance level of 5% in all our analyses. Data were analysed using R (Version 3.5.1, www.r-project.org). Details on sample size are presented in the supplementary materials (Supplement S1).

Results

Participants

The recruitment process is summarized in a CONSORT flow diagram (Supplement Figure S1). In the cross-sectional part, 35 persons were included in the HA group, 33 in the HS group and 79 in the SR group. Eighty-six percent of the SR group were obese, 70% were hypertensive, 56% suffered from dyslipidaemia, 41% were diabetics and 34% were smokers (Supplement Table S1). The participants` characteristics of all three groups including CV risk factors, PA and CRF data as well as the vascular indices are presented in Table 1.

Cross-sectional part

Overall higher CRF was significantly associated with lower central PWV (p<0.001) and VO_{2max} explained 18% of the variance in PWV in all participants after adjustment for age and sex (Supplement Figure S2). An increase of 10 ml/min/kg in VO_{2max} was associated with a decrease of PWV by 0.8 m/s (p<0.001). PWV increased with an increasing number of risk factors (p for Trend <0.01; Supplement Figure S3). In the between group comparison, PWV was highest in

Table 1. Participants` characteristics

	HA (n=35)	HS (n=33)	SR (n=79)	*p*
Clinical data				
Age, yr	60±7	59±7	58±6	0.325
Female sex, n (%)	17 (49)	24 (73)	41 (52)	0.080*
Height, cm	171±7.7	168±8.8	169±8.0	0.403
Body mass, kg	64.4±6.6	70.7±10.2	95.5±13.9	<0.001
BMI, kg/m^2	22.2±1.7	24.9±2.5	33.4±4.0	<0.001
WC, cm	82±7	89±9	112±12	<0.001
HR, bpm	62±8	77±12	79±11	<0.001
SBP at rest, mmHg	128±16	128±15	132±15	0.317
DBP at rest, mmHg	78±8	81±8	88±10	<0.001
PP at rest, mmHg	50±12	46±11	44±12	0.052
24H SBP, mmHg	119±6	121±7	130±11	<0.001
24H DBP, mmHg	76±6	76±6	81±8	<0.001
Fasting glucose, mmol/l	4.6±0.4	4.7±0.5	5.7±1.8	<0.001
Triglyceride, mmol/l	0.92±0.28	1.09±0.31	1.80±1.11	<0.001
HDL, mmol/l	1.99±0.41	1.69±0.38	1.30±0.32	<0.001
LDL, mmol/l	2.85±0.75	3.2±0.83	3.23±0.79	0.064
hsCRP, mg/l	0.9±1.0	1.7±2.3	3.7±4.2	<0.001
Physical activity and fitness				
Total PA, MET/week	171±8.4	135±56	126±55	<0.001
Sport activity, MET/week	47±37	2±2	1±3	<0.001
Steps per day, n	13800±4629	10222±3213	9028±3283	<0.001
Walking per day, min	31±25	19±13	14±11	<0.001
VO_{2max}, ml/min/kg	42.6±8.2	29.7±4.0	26.1±4.4	<0.001
Arterial Stiffness				
Central PWV, m/s	7.0±1.1	7.5±1.6	8.2±1.4	<0.001

Abbreviations: HA, healthy active; HS, healthy sedentary; SR, sedentary at risk; BMI, body mass index; WC, waist circumference; HF, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density-lipoprotein, LDL, low-density-lipoprotein; hsCRP, high-sensitivity C-reactive Protein; PA, physical activity; MET, metabolic equivalent; VO_{2max}, maximal oxygen uptake; PWV pulse wave velocity. Data are mean ± standard deviation. ANOVA was used to calculate overall group differences.
*Chi-squared test was used

SR (8.2±1.4 m/s) compared to HS (8.2±1.4 m/s) and HA (7.0±1.1 m/s; Table 2). ANOVA revealed a significant overall group difference (p<0.001). Multiple linear regression revealed significant differences in PWV between all groups (Figure 1A; Supplement Table S2).

Table 2. Population characteristics in sedentary at risk before and after HIIT

Outcome	**Adjusted difference***	Lower CI (95%)	Upper CI (95%)	***p***
Anthropometric data				
Weight, kg	**-0.4**	-1.5	0.7	**0.45**
BMI, kg/m2	**-0.2**	-0.6	0.2	**0.38**
WC, cm	**-1.2**	-4.2	1.7	**0.41**
Fat mass, %	**-2.0**	-3.6	-0.5	**0.01**
Muscle mass, %	**1.0**	0.2	1.9	**0.02**
HR, bpm	**-2.9**	-7.6	1.7	**0.21**
SBP at rest, mmHg	**-1.0**	-6.0	4.7	**0.80**
DBP at rest, mmHg	**1.6**	-2.2	5.5	**0.39**
Cardiorespiratory fitness				
VO_{2max}, ml/min/kg	**3.4**	2.5	4.3	**<0.001**
Arterial Stiffness				
Central PWV, m/s	**0.1**	-0.3	0.6	**0.60**
Central PWV†, m/s	**0.2**	-0.2	0.6	**0.29**

Abbreviations: CI, confidence interval; BMI, body mass index; WC, waist circumference; HF, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; PWV, pulse wave velocity. * Analysis of covariance was used to detect differences of post- versus pre-intervention values between intervention and control condition. †Additional adjustment for delta of systolic blood pressure (pre- minus post-value).

Interventional part

The final analysis was performed in 38 persons of the intervention group and 30 persons of the control group (Supplement Figure S1). After 12 weeks of HIIT, CRF improved significantly, but this was not accompanied by significant changes in central PWV (Table 2; Figure 1B). Adjustment for differences in systolic BP (pre- to post-training) did not change these results. Pre- and post- values for all measurements in the intervention and the control group are

presented in Supplement Table S3. Main determinants for the adaptations of PWV after HIIT were the baseline PWV (p<0.001) and changes in systolic BP (p<0.018). The association of changes in systolic BP with changes in central PWV is shown in Supplement Figure S4.

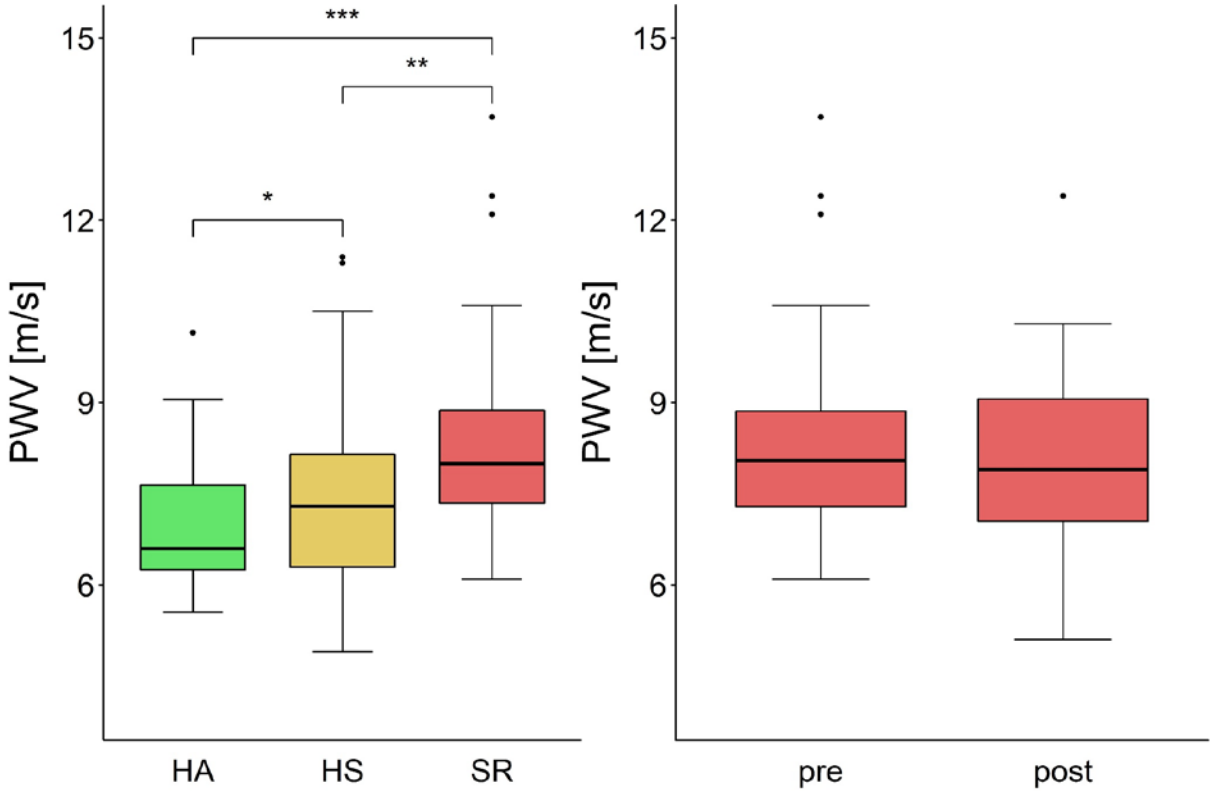

Figure 1: Central pulse wave velocity in the cross-sectional (A) and interventional (B) approach.

Abbreviations: HA, healthy active; HS, healthy sedentary; SR, sedentary at risk; *p<0.05; **p<0.01; ***p<0.001.

Discussion

Our study results demonstrate the importance of long-term PA and the limited impact of short-term exercise training on large artery stiffness in an older population. Long-term PA was associated with lower central PWV even in the absence of CV risk factors. PWV was higher in SR compared to HS. Overall, higher VO_{2max} and fewer CV risk factors were associated with lower PWV. Most importantly, 12-weeks of HIIT did not reduce PWV in elderly at increased CV risk.

Aging is characterized by continuous remodelling of the arterial wall and Vaitkevicius et al. were the first to suggest that higher CRF may mitigate stiffening of the aging arterial tree[17]. In our study, with every 10 ml/min/kg increase in VO_{2max}, PWV dropped by 0.8m/s. Active participants presented with 0.5 m/s lower central PWV than their sedentary counterparts. An increase of 1 m/s in central PWV has been associated with a 15% risk increase in CV and all-cause mortality[3]. Thus, our cross-sectional findings indicate an 8% risk increase attributable to a sedentary lifestyle even in healthy elderly.

Whether short-term exercise can postpone or even reverse the age- and disease- related progression of AST remained unresolved to date. HIIT has been shown to be effective in reducing CV risk in patients with metabolic syndrome[9]. Only few studies have evaluated the effects of HIIT on central PWV and these produced conflicting results. While Ciolac et al.[11] and Guimarães et al.[10] were able to show a reduction of AST in response to HIIT in young individuals with increased CV risk, these findings could not be reproduced in healthy older persons by Kim et al.[18]. Our study is the first to examine the effects of 12 weeks of HIIT in sedentary elderly with CV disease, demonstrating that high-intensity exercise training does not improve large artery stiffness in older individuals. Several systematic reviews and meta-analyses with inconsistent findings exist on the effects of exercise interventions on AST, but none of them set focus on elderly people[19-22]. Our results indicate a reduced vascular adaptability in older individuals and highlight the pivotal role of age when assessing the effect of exercise training on AST.

This is underlined by our cross-sectional data showing that sustained long-term habitual PA is associated with better vascular function. This raises the question whether a threshold exercise duration may exist in order to achieve improvement of AST in elderly with CV disease. The two studies reporting reduced PWV after HIIT lasted 16 weeks but were conducted in younger individuals[10, 11]. In sedentary elderly persons, one year of aerobic exercise at moderate intensity did not reduce AST[8]. Moreover, it has previously been argued that significant improvements in VO_{2max} are prerequisite for relevant reductions in PWV after short-term exercise[20]. In our study however, HIIT induced a significant increase of CRF without improving PWV in elderly with CV disease. In conjunction with the previous literature and our current findings it becomes evident that sustained long-term PA may be needed to improve, postpone or even reverse the increased PWV in elderly with CV disease.

Our results contradict Montero et al. who found that a reduction in central PWV was dependent on concomitant reductions in systolic BP[22]. From a physiological point of view, close associations between BP and PWV are to be expected[23]. Indeed, our intervention group showed significant associations between changes in central PWV and systolic BP. In addition, 46% of our participants in the SR group were already treated for hypertension. In elderly persons pretreated for high BP, additional HIIT may have limited effects on further BP reductions, and the arterial capacity to adapt to exercise stimuli may be diminished.

To put our findings into perspective and add to the debate on the effects of exercise on AST, possible underlying mechanisms need to be discussed. Vascular wall integrity is defined by functional as well as structural properties of the arterial wall[24]. Functional changes are largely dependent on vascular smooth muscle tone which is regulated locally by endothelial function. Structural properties depend, in large part, on the collagen and elastin composition of the vascular wall. Recently, the role of exercise as a modulator of AST in the context of the potential mechanisms involved has been adressed[25]. Though exercise affects functional as well as structural components of the arterial wall, Sacre et al. suggested that longer-term exercise would be needed to provoke structural changes in the arterial wall[25]. Our results support this postulation. This seems to be the case for older age in particular, where long lasting structural elastin degradation and collagen deposition cannot be reversed by relative short-term high-intensity exercise interventions.

Few limitations have to be addressed. An unbalanced sex distribution is evident in HS. This may have tempered between group differences. However, sex does not account for normal values of PWV according to current recommendations[26]. Our results were adjusted for sex to minimize potential confounding and improve the proportion of explained variance. In addition, only sedentary persons at increased CV risk performed HIIT, as this group was expected to benefit most from aerobic exercise. Our Patients were characterized by a number of treated and untreated CV diseases that may have differential impacts on the arterial wall. We believe this represents the real life setting, as elderly persons are increasingly prone to comorbidities.

Conclusion

In conclusion, CRF is a main determinant of central PWV in an aging population. Our results demonstrate that long-term active compared to sedentary lifestyle is associated with lower AST even in healthy elderly. This suggests that age- and disease- related vascular stiffening and the associated worse CV outcome can be postponed by long-term regular PA. Short-term exercise, even at higher intensities, cannot improve arterial stiffening in sedentary elderly with increased CV risk. Exercise-induced reductions of AST seem to depend on a concomitant decrease of BP. The results of our study shed light on the influence of long-term PA, CRF and the effects of short-term exercise on arterial stiffening as treatment options for CV disease prevention in an older population.

Conflict of interest

None.

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26. Reference Values for Arterial Stiffness Collaboration. Determinants of pulse wave velocity in healthy people and in the presence of cardiovascular risk factors: 'establishing normal and reference values'. Eur Heart J 2010;**31**(19):2338-50.

SUPPLEMENTARY MATERIAL

Physical activity

PA was obtained combining self-reported and objective techniques. During medical examination, participants gave information on past and current PA habits as well as regular sports participation within the last 10 years. A short form of the Freiburg Questionnaire of Physical Activity served to calculate metabolic equivalents (METs) based on the Ainsworth Compendium[1, 2]. This validated questionnaire allows for estimation of total METs per week as well as METs achieved during sport activities. Objective measurement of daily PA was assured by wearing an Aipermotion 440 accelerometer (Aipermon GmbH, Germany) for six consecutive days. Steps and minutes of walking per day were calculated using the AiperView 440 and ActiCoach MPAT2Viewer Software (Aipermon GmbH, Germany).

Cardiorespiratory fitness

Maximal aerobic capacity was obtained by individual ramp protocols on a treadmill ergometry as recommended previously[3]. The protocol was chosen according to the estimation of the participant`s exercise capacity and were set to reach a test duration of 8-12 minutes as suggested by the American College of Sport Medicine. VO_{2max} and maximum heart rate were recorded for each individual. Gas exchange was assessed using a calibrated breath-by-breath spirometric system (Metalyzer® 3B, MetaSoft®, CORTEX Biophysik GmbH, Germany).

Anthropometry and blood sampling

Anthropometric measurements and blood sampling were conducted in the morning under fasting conditions. Body height and body weight were measured to calculate body mass index (kg/m^2). Body composition was assessed using a standard bio-impedance analyser (InBody 720, Inbody Co., Ltd, Korea). Blood was drawn by venepuncture of the brachial vein in lithium heparin tubes. Platelet-free plasma was separated by centrifugation (3000g at room temperature for 10 minutes), pipetted in aliquots and stored at -80°C for subsequent measurement. High-sensitivity c-reactive protein (hsCRP) was assayed by turbidimetry. 24-hour ambulatory BP was obtained by the use of an oscillometric cuff-based

sphygmomanometer on the right arm (Mobil-O-Graph®, I.E.M GmbH, Germany). Recordings were performed every 20 minutes during daytime and every 30 minutes during nighttime.

Sample Size Calculation

Details on sample size calculation have previously been published in the study protocol and were performed for the cross-sectional and interventional approach separately[4]. For the cross-sectional part we assumed that the expected central PWV corresponds to 8.5 m/s, 9.5 m/s and 11.5 m/s for HA, HS and SR, respectively, and that the standard deviation given any particular group is 1.5 m/s[5, 6]. Central PWV was the main outcome and an 80% power on a 2-sided significance level of 0.05 was targeted. This resulted in 36 participants needed for each group in the cross-sectional approach. For the interventional part of the study, we assumed that the expected difference in central PWV after 12 weeks between SR in the intervention and those in the control group is 1.0 m/s and that the standard deviation is 1.5 m/s[7] . This led to the calculation of a minimum number of 36 participants. Taking dropouts into account we aimed to reach 40 subjects in the HA and 10 HS group and 80 persons in the SR group (40 intervention and 40 controls). For sample size calculation, we used the POWER and GLMPOWER procedure in SAS 9.3 (SAS Institute Inc., Cary, NC).

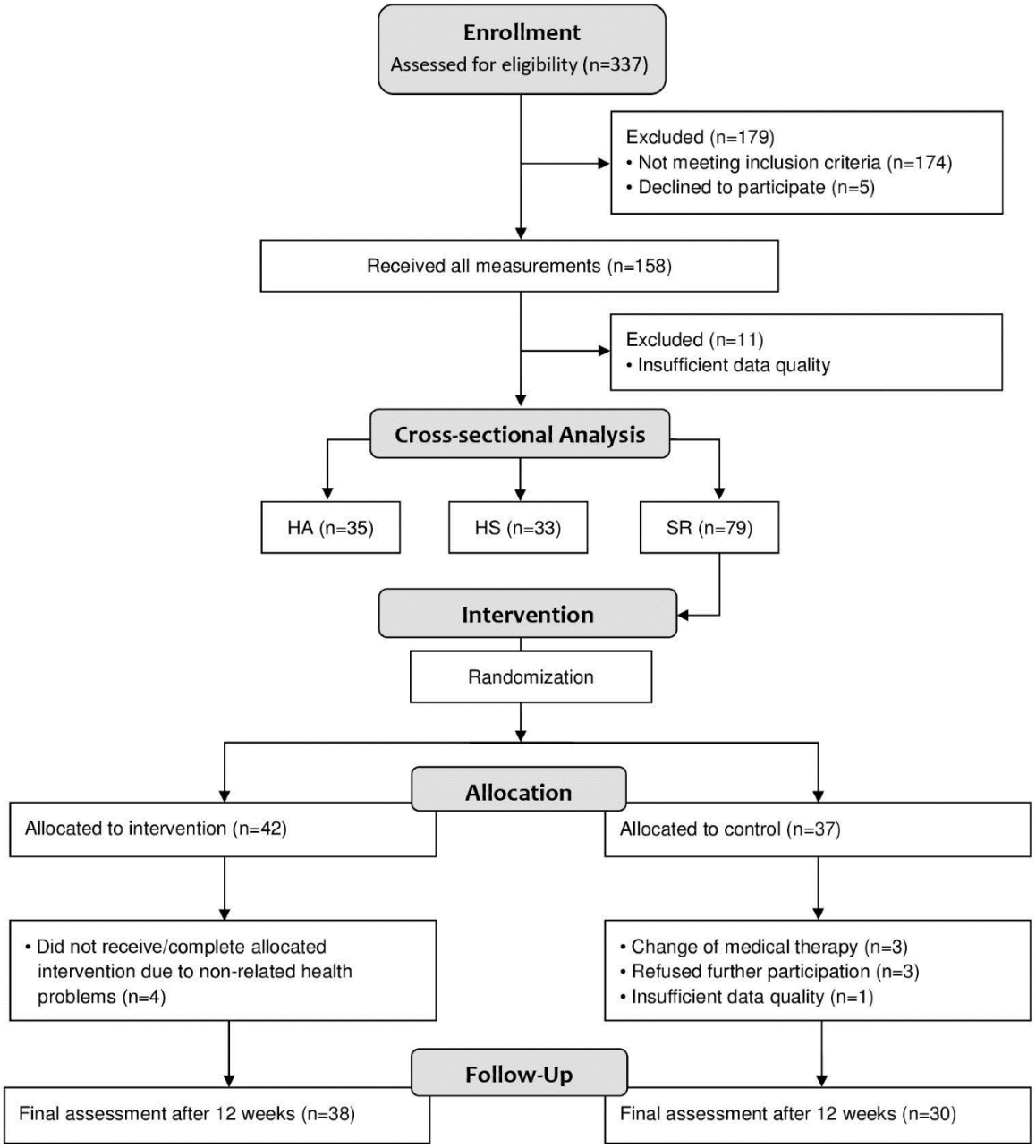

Figure S1. Flow-chart

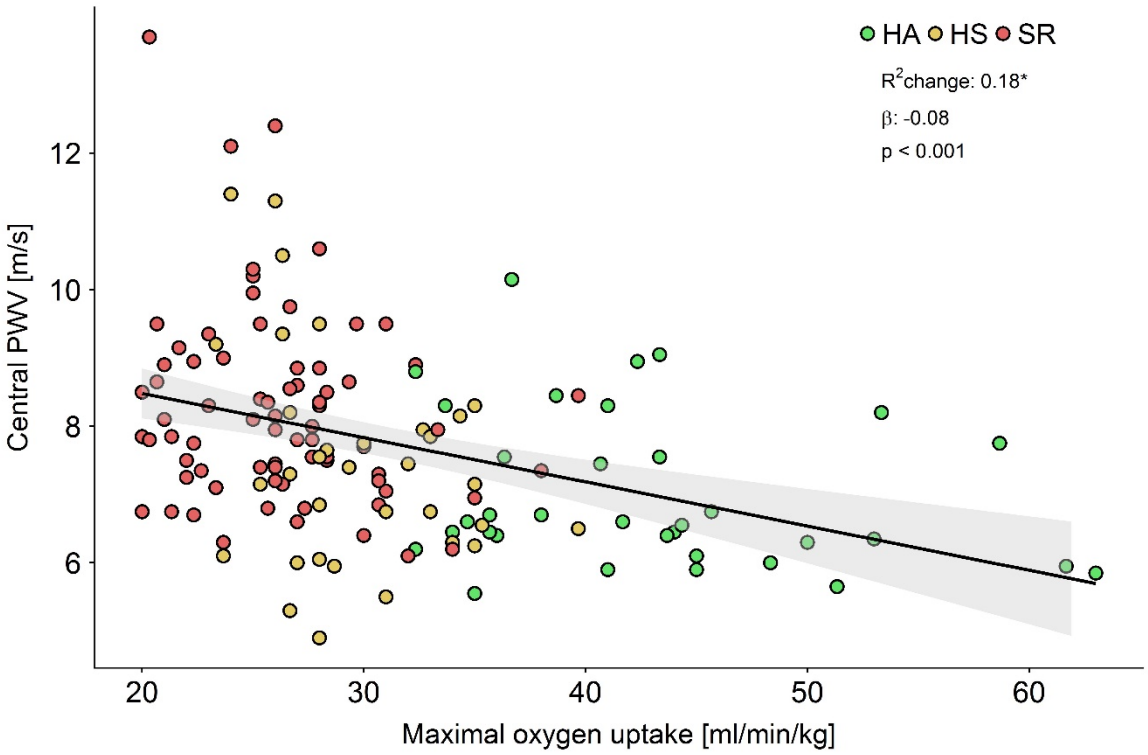

Figure S2.

Scatterplot showing maximal oxygen uptake by central pulse wave velocity (PWV) for healthy active (HA) and sedentary (HS) as well as sedentary at risk (SR). Regression line and 95% confidence intervall of mean standard deviation are visualized. Multiple linear regression was adjusted for age and sex.

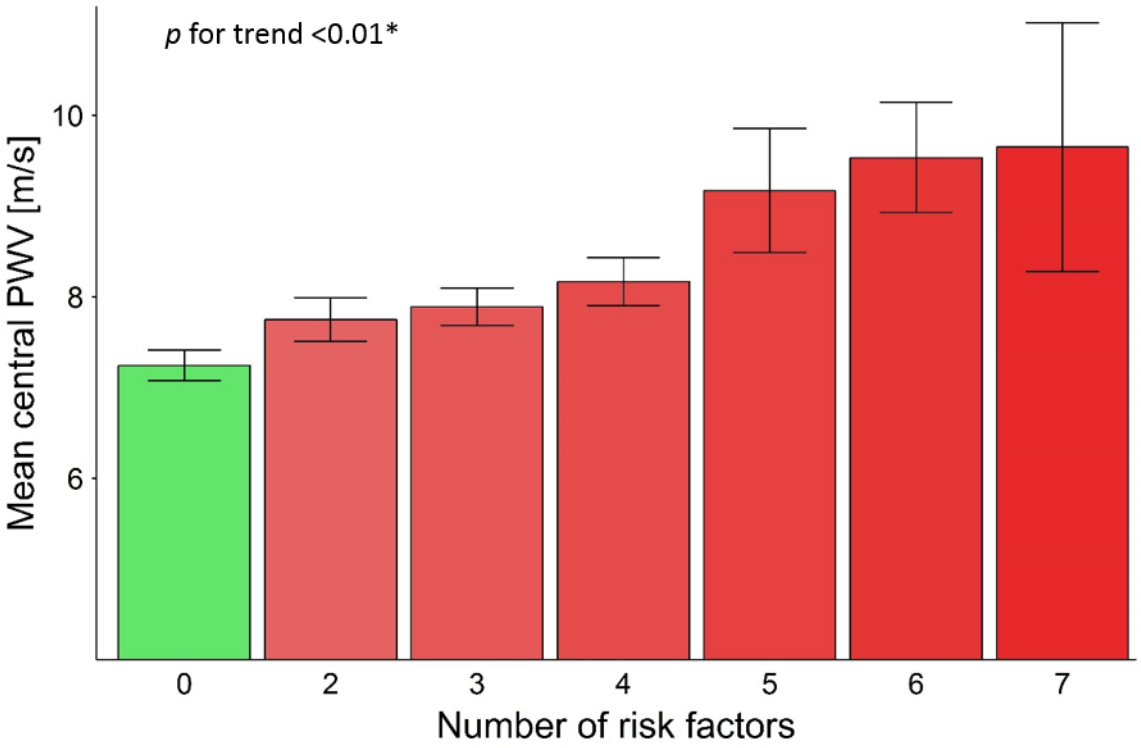

Figure S3. **Number of risk factors in participants and the corresponding mean central pulse wave velocity (PWV).**

*Jonckheere Trend Test.

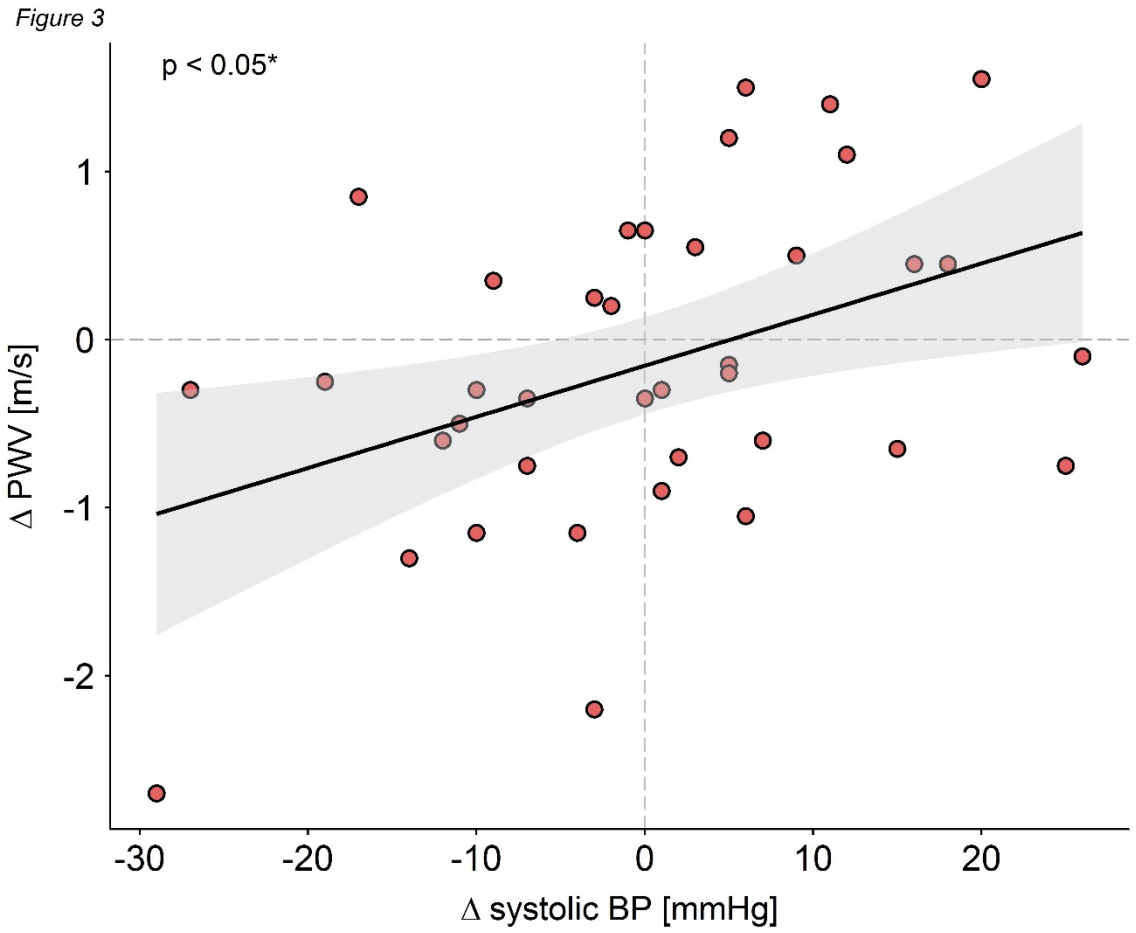

Figure S4. Regression line and 95% confidence interval of standard deviation are visualized. Deltas were calculated subtracting pre from post value. *Multiple linear regression adjusted for age and sex, baseline central PWV and body mass index. Abbreviations: BP, blood pressure; PWV, pulse wave velocity.

Table S1. Risk factors in sedentary at risk

	n	%
Obesity	**68**	**86**
High Blood Pressure	**48**	**61**
SBP ≥ 140 mmHg or DBP ≥ 90 mmHg (24 hour)	17	32
Antihypertensive medication	36	46
Dyslipidaemia	**44**	**56**
LDL > 4.9 mmol/l	2	3
HDL < 1.0 mmol/l (male) or < 1.2 mmol/l (female)	18	23
Triglycerides > 1.7 mmol/l	29	37
Cholesterol lowering medication	14	18
Diabetes	**32**	**41**
Fasting glucose ≥ 5.6 mmol/l	32	41
Antidiabetic medication	8	10
Smoking	**27**	**34**

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein, HDL, high-density lipoprotein.

Table S2. Between group differences in central pulse wave velocity

	Model	adj. R^2	Mean difference (95% CI)	p
HA to HS	1	0.34	0.64 (0.07, 1.20)	0.027
	2	0.38	0.63 (0.08, 1.18)	0.026
HS to SR	1	0.26	0.79 (0.25, 1.32)	0.004
	2	0.28	0.70 (0.17, 1.23)	0.010
HA to SR	1	0.32	1.37 (0.90, 1.84)	<0.001
	2	0.33	1.33 (0.86; 1.81)	<0.001

Abbreviations: PWV, pulse wave velocity adj., adjusted; CI, confidence interval; HA, healthy active; HS, healthy sedentary; SR, sedentary at risk. Multiple linear regression for group differences central pulse wave velocity (m/s). Model 1: adjusted for age and sex Model 2: adjusted for age, sex and systolic blood pressure

Table S3. Population characteristics in sedentary at risk before and after HIIT

	Intervention (n=38)		Control (n=30)	
Outcome	Pre	Post	Pre	Post
Clinical data				
Age, yr	58±5		57±6	
Female sex, n (%)	18		19	
Weight, kg	95.1±12.3	93.9±12.6	94.4±14.8	93.6±14.5
BMI, kg/m^2	33.3±3.0	32.8±3.2	33.1±5.1	32.8±5.0
WC, cm	111±9	109±10	110±14	110±14
Fat mass, %	40±8	38±8	42±8	42±7
Muscle mass, %	32±7	33±7	30±6	30±5
HF, bpm	79±12	74±11	76±9	75±11
SBP at rest, mmHg	133±14	134±12	128±14	131±15
DBP at rest, mmHg	88±10	87±7	86±10	85±11
24h SBP, mmHg	130±10	132±12	128±10	126±11
24h DBP, mmHg	82±7	83±8	79±8	79±9
Fasting glucose, mmol/l	5.8±2.2	5.7±1.8	5.5±1.1	5.5±1.1
Triglyceride, mmol/l	1.82±1.03	1.87±1.13	1.62±0.79	1.82±1.00
HDL, mmol/l	1.29±0.3	1.29±0.28	1.37±0.31	1.35±0.34
LDL, mmol/l	3.34±0.83	3.08±0.90	3.03±0.69	2.92±0.78
hsCRP, mg/l	3.3±2.5	3.0±2.2	4.1±6.1	4.4±7.8
Cardiorespiratory fitness				
VO_{2max}, ml/min/kg	26.4±3.9	28.6±1.1	26.2±5.1	25.1±4.2
Arterial Stiffness				
Central PWV, m/s	8.2±1.2	8.1±1.1	8.2±1.6	7.9±1.6

Abbreviations: SD, standard deviation; BMI, body mass index; WC, waist circumference; HF, heart frequency; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive Protein; PWV, pulse wave velocity. Data are mean ± standard deviation. †Additional adjustment for delta of systolic blood pressure.

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3. Myers J, Bellin D. Ramp exercise protocols for clinical and cardiopulmonary exercise testing. Sports Med 2000;**30**(1):23-9.
4. Streese L, Deiseroth A, Schafer J, Schmidt-Trucksass A, Hanssen H. Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population: The ExAMIN AGE Study. Front Physiol 2018;**9**:116.
5. Gando Y, Yamamoto K, Murakami H, Ohmori Y, Kawakami R, Sanada K, Higuchi M, Tabata I, Miyachi M. Longer Time Spent in Light Physical Activity Is Associated With Reduced Arterial Stiffness in Older Adults. Hypertension 2010;**56**(3):540-546.
6. Tedesco MA, Natale F, Di Salvo G, Caputo S, Capasso M, Calabro R. Effects of coexisting hypertension and type II diabetes mellitus on arterial stiffness. J Hum Hypertens 2004;**18**(7):469-73.
7. Madden KM, Lockhart C, Cuff D, Potter TF, Meneilly GS. Short-Term Aerobic Exercise Reduces Arterial Stiffness in Older Adults With Type 2 Diabetes, Hypertension, and Hypercholesterolemia. Diabetes Care 2009;**32**(8):1531-1535.

9.3 Curriculum vitae

PERSONAL INFORMATION

Date of birth:	July 5th 1989
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EDUCATION

11/2015-07/2019	**PhD Program in Health Sciences (PPHS), University of Basel,** Basel, PhD supervisor: Prof. Dr. Henner Hanssen
10/2013-09/2015	**Master of Science Sport and Movement Gerontology,** German Sports University Cologne, Cologne
10/2010-09/2013	**Bachelor of Science in Sports and Performance,** German Sports University Cologne, Cologne

PROFESSIONAL EXPERIENCE

Since 07/2019	**Postdoc** at the Department of Sport, Exercise and Health (DSBG), Section Preventive Sports Medicine and Systems Physiology, University of Basel
11/2015-07/2019	**PhD** at the Department of Sport, Exercise and Health (DSBG), Section Preventive Sports Medicine and Systems Physiology, University of Basel Study coordinator of the EXAMIN AGE Study
09/2013-09/2015	**Personal Health Coach and Performance Analyst**

INSTITUTIONAL RESPONSIBILITIES

Since 07/2017	**Member of the "Departementsversammlung"** of the Department of Sport, Exercise and Health, representing Group III (PhDs and Post-Docs)

RESEARCH PROJECTS

Since 05/2018	**Support to develop Next generation sequencing** from circulating mRNA
Since 01/2018	**COMPLETE-Health Study:** Cardio-Pulmonary Exercise Testing (retinal vascular analysis)
Since 08/2017	**Analysis of the retinal vessel wall** (study manager and coordinator)
Since 03/2016	**ExTRA RISK Study:** Cross-sectional and interventional study with rheumatic patients (retinal vascular analysis)
Since 11/2015	**EXAMIN AGE Study:** Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population (study manager, responsible for all tests especially for retinal vascular analysis); SNSF project funding (#32003B_ 159518/ 1)

SUPERVISION OF STUDENTS

Master students (13): Andri Rickli, Andri Burri, Maria Thomann, Eveline Schärli, Lino Cerletti, Raphael Schoch, Jessica Dünki, Christina Schulz, Tamara Geiger, Gilles Nève, Silvan Lenzlinger, Benjamin Hirschi, Romy Wüst

Bachelor students (12): Isabel Hofer, Elena Westerhuis, Melanie Thut, Aurelia Altherr, Eveline Schärli, Fiorenzo Pedrocchi, Leda Maffei, Ishbel Lomax, Geraldine Studer, Lukas Brawand, Nadine Schwegler, Tina Müller

TEACHING ACTIVITIES

Since 02/2018	**Lecturer** of "Cardiovascular Diagnostics", Department of Sport, Exercise and Health (DSBG), Section Preventive Sports Medicine and Systems Physiology, University of Basel
Since 02/2018	**Lecturer and teaching coordinator** of "Hands-on Vascular Physiology", Department of Sport, Exercise and Health (DSBG), Section Preventive Sports Medicine and Systems Physiology, University of Basel
Since 01/2017	**Lecturer** in "Bewegungs- und Gefässphysiologie", Department of Sport, Exercise and Health (DSBG), Section Preventive Sports Medicine and Systems Physiology, University of Basel

MEMBERSHIP IN BOARDS

Swiss Society of Sports Science (SGS)

Swiss Society of Microcirculation and Vascular Research (SSMVR)

European Association of Preventive Cardiology (EAPC)

European College of Sport Science (ECSS)

European Society of Cardiology (ESC)

European Society of Microcirculation (ESM)

The International Society of Exercise Immunology (ISEI)

GRANTS

05/2018	**Top-up Stipend Award** from PPHS for next generation sequencing of micro RNA from circulating blood (CHF 11250,-),
12/2017	**Grant** from PPHS "Invite your expert" (CHF 1050,-),

REVIEWER WORK

Atherosclerosis

Current Issues in Sport Science (CISS)

LANGUAGES

German: mother language

English: fluent in writing and speaking

COMPUTER SKILLS

Software MS Office (Word, Excel, PowerPoint), R, SPSS

PUBLICATION LIST

Streese, L., Kotliar, K., Deiseroth, A., Infanger, D., Vilser, W. and Hanssen, H. (submitted). «Retinal endothelial function, physical fitness and cardiovascular risk: a diagnostic challenge», Frontiers in Physiology, 10/831. doi: 10.3389/fphys.2019.00831.

Streese, L., Khan, A.W., Deiseroth, A., Hussain, S., Suades, R., Tiaden, A., Kyburz, D., Cosentino, F. and Hanssen, H. (2019) «High-intensity interval training modulates retinal microvascular phenotype and DNA methylation of $p66^{Shc}$ gene: a randomized controlled trial (EXAMIN AGE)», European Heart Journal, in press.

Streese, L., Königstein, K., Goricki, L., Infanger, D., Wölnerhanssen, B., Peters, T., Schmidt-Trucksäss, A. and Hanssen, H. (2019) «Short- and Long-Term Effects of Bariatric Surgery on Vascular Phenotype», Obesity Surgery, 29(4):1301-1308. doi: 10.1007/s11695-018-03679-2.

Streese, L., Deiseroth, A., Schäfer, J., Schmidt-Trucksäss, A. and Hanssen, H. (2018) «Exercise, Arterial Crosstalk-Modulation, and Inflammation in an Aging Population: The EXAMIN AGE Study», Frontiers in Physiology, 116/9. doi: 10.3389/fphys.2018.00116.

Pizzera, A., Hohmann, T., **Streese, L.**, Habbig, A. and Raab, M. (2017) «Long-term effects of acoustic reafference training (ART)», European journal of sport science, 17(10), S. 1279-1288. doi: 10.1080/17461391.2017.1381767.

Baum, K., Hoffmann, U. and **Streese, L.** (2016) «Exercise - induced Hypoalgesia in Physically Active Men Following Different Exhaustive Cycle-ergometries», Journal of Applied Life Sciences International, 8(1), S. 1-8. Available at: http://www.journalrepository.org/media/journals/JALSI_40/2016/Sep/Baum812016JALSI28731.pdf.

Kennel, C., **Streese, L.**, Pizzera, A., Justen, C., Hohmann, T. and Raab, M. (2015) «Auditory reafferences: the influence of real-time feedback on movement control», Frontiers in Psychology. Frontiers Research Foundation, 6, S. 69. doi: 10.3389/fpsyg.2015.00069.

Submitted manuscripts:

Streese, L., Kotliar, K., Deiseroth, A., Infanger, D., Gugleta, K., Schmaderer, C. and Hanssen, H. (submitted). «Retinal endothelial function in cardiovascular risk patients: a randomized controlled exercise trial», submitted to The Scandinavian Journal of Medicine & Science in Sports (March 2019).

Streese, L., Khan, A.W., Deiseroth, A., Hussain, S., Suades, R., Tiaden, A., Kyburz, D., Cosentino, F. and Hanssen, H. (submitted). « Association of physical activity and cardiovascular risk with retinal microvascular phenotype: $p66^{Shc}$ expression as a putative mechanistic link», submitted to European Journal of Preventive Cardiology (March 2019).

Arne, D., **Streese, L.**, Köchli, S., Wuest, R., Infanger, D., Schmidt-Trucksaess, A. and Hanssen, H. (submitted). «Exercise and arterial stiffness in the elderly: A combined cross-sectional and randomized controlled trial (EXAMIN AGE)», submitted to Age and Ageing (March 2019).

CONFERENCE PARTICIPATIONS

2019 Poster presentation at "*ESM-EVBO*", Maastricht, Netherlands.

2019 Poster presentation at "*EuroPrevent*", Lisbon, Portugal.

2019 Oral presentation at "*11. Jahrestagung der Sportwissenschaftlichen Gesellschaft der Schweiz"*, Fribourg, Switzerland.

2018 Poster presentation at "*ESC congress*", Munich, Germany.

2018 Active participation at "*EuroPrevent*", Ljubljana, Slovenia.

2018 Active participation at "*10. Jahrestagung der Sportwissenschaftlichen Gesellschaft der Schweiz*", Magglingen, Switzerland.

2017 Oral presentation at "*9. Jahrestagung der Sportwissenschaftlichen Gesellschaft der Schweiz*", Zurich, Switzerland.

2017 Active participation at the international symposium "*Cardiometabolic risk and vascular disease*", Stockholm, Sweden.

2016 Oral presentation at "*Deutscher Sportärztekongress*", Frankfurt, Germany.

9.4 Graduate Education

Course	ECTS
Essentials in Health Research Methodology; University of Basel, Clinical Trail Unit	2
Good Scientific Practice; University of Basel, Helga Nolte	1
Introduction to statistics with R; University of Basel, Nathaniel Phillips	2
CardioLung 2017: Updates in Cardiovascular and Pulmonary Pathophysiology; University of Pisa, Prof. Carlo Palombo	6
Medical Decision Making; University of Lucerne, Brendan Delaney and Olga Kostopoulou	1
Lehren und Lernen in der Erwachsenenbildung; University of Basel, Falk Scheidig	3
Good clinical practice; University of Basel, Christian Burri	1
1st Summer School of the European Society for Microcirculation; European Society of Microcirculation, Prof. Henning Morawietz	2
GESIS Summer School in Survey Methodology (Meta-Analysis), Leibzig-Institut für Sozialwissenschaften	2
Presenting and discussion in academic and professional settings; Sprachzentrum University of Basel, Stephan Meyer	2
Total	22