THE EFFECT OF EXERCISE AND ACE INHIBITION ON ANGIOTENSIN II

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THE EFFECT OF EXERCISE AND ACE INHIBITION ON ANGIOTENSIN II

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Summary

Within the regulation of blood pressure, the renin-angiotensin system (RAS) plays an essential role with AngII causing vasoconstriction, which increases the blood pressure. AngII is formed after *ACE* ar cleaved by AngI. We hypothesize that the blunting of AngII production with concomitant exercise reduces improvements in metabolic fitness by removing an important stimulus for capillary growth in exercised muscles. The aim of this research was to expose the pathway by which AngII and *ACE* are implicated in exercise-induced capillary growth of human muscle. We show that highest increases in indices of metabolic strain are observed after high intense exercise bouts and that the increase in AngII is *ACE* genotype dependent. Furthermore, subjects with the *ACE*-II genotype had a significant higher capillary perfusion in the finger after exercise. Our findings of our *ACE* inhibition study support the view that an angiotensin-regulated mechanism affects the hypoxia-specific gene response in peripheral muscle to endurance exercise and support the notion that the muscle's transcript regulation by *ACE* inhibition is related to muscle oxygenation during exercise. This indicates that there is a shift in the activation of the gene program from muscle fibres to the surrounding interstitium after *ACE* inhibition. In a training study we identified a correlation between increases in VO_2max and the changes in capillary-to-fibre-ratio. We further observed that variability in the cardiovascular response, based on VO_2max and heart rate at rest, was related to the *ACE* I/D genotype. Our findings that physiological improvements after exercise are dependent on *ACE* levels, both due to different *ACE* genotypes and to taking *ACE* inhibitors could have clinical repercussions. It may explain the individual variation in the response to exercise rehabilitation in aerobic power and oxygen uptake. These differences in exercise-induced improvements have rarely been valued in pharmacological studies of hypertension and exercise rehabilitation.

Chapter 1: Introduction

High blood pressure (hypertension) indicates the deterioration of metabolic fitness and is one of the most frequent metabolic risk factors for disease in Western countries (North of England Hypertension Guideline Development Group, 2004). The risk to develop hypertension and associated metabolic syndromes increases with age (Burt et al. 1995). Consequently, hypertension is a major target of the current pharmacological treatments producing yearly expenses in the order of 100s of million Euros in middle-sized European countries alone (North of England Hypertension Guideline Development Group, 2004)

Amongst the factors that regulate blood pressure, the renin-angiotensin system (RAS) plays an essential role. Renin is produced in the kidneys when the blood pressure at the juxtaglomerular apparatus decreases (< 90 mmHg systolic blood pressure) or when there is a decreased sodium chloride delivery to the macula densa (Silbernagl et al. 2001). Angiotensinogen, a plasma protein synthesized in the liver, is cleaved by renin and forms angiotensin I (AngI). AngI appears to have no biological activity but circulating AngI is cleaved by the angiotensin-converting enzyme (*ACE*) to form angiotensin II (AngII). AngII causes vasoconstriction; water and salt retention, stimulates releases of aldosterone from the adrenal cortex and stimulates capillary growth in blood flow-induced angiogenesis in skeletal muscle (Petersen and Greene, 2007).

Angiotensin-converting enzyme (ACE)

ACE is a dipeptidipeptidyl-carbo-peptidase which is located in plasma, pulmonary and vascular endothelial cells and the heart (Brewster and Perazella 2004) and has the highest density in the lungs. *ACE* is secreted by pulmonary and renal endothelial cells and mediates extracellular volume and arterial vasoconstriction. Besides of converting AngI to AngII, *ACE* inhibits bradykinin, a potent vasodilator which can stimulate the release of vasodilating prostaglandins and nitric oxide. Within an individual the plasma *ACE* levels are very stable (Alhenc-Gelas et al. 1991), however there are remarkable interindividual variations (Cambien et al. 1988, Tiret et al. 1992). These variations are due to the existence of polymorphism in the *ACE* gene and environmental factors such as physical activity and thermal stress (Staessen et al. 1987); Kosunen J Appl Physiol 41(3), 1976; Danser et al. 2007). Besides *ACE* there also exists *ACE2* which is mainly expressed in vascular endothelial cells of the heart and kidney. The general catalytic function of *ACE2* is equivalent as that of *ACE* and about 40% of the catalytic site of *ACE2* correspondents to ACE. However *ACE2* catalyses the specific petidyl cleavages including cleavages of the carbosyl terminal phenylalanine residue of AngII into the vasodilator angiotensin-(1-9). So *ACE2* can function antagonistically to *ACE* by destroying AngII and creating angiotensin-(1-9) (Vickers et al. 2002; Donoghe et al. 2000).

A major strategy of the current pharmacological treatments against hypertension consists in the lowering of serum AngII levels by pharmacological inhibition of ACE. This medication develops a generally positive effect on mortality and *ACE* inhibitors are among the most prescribed medication worldwide (Danser et al. 2007).

Within intron 16 on the *ACE* gene people have an insertion (I) or deletion (D) of the 287-base pair which results in three different genotypes: ACE-II, ACE-DD and ACE-ID. Subjects with the presence of I allele in the ACE-ID and ACE-II genotypes have respectively approximately 30% and 60% lower plasma *ACE* activity (Rigat et al. 1990) and also higher tissue activity levels than ACE-DD subjects (Costerousse et al. 1993; Danser et al. 1995). It is known that people with the D-allele have a higher chance of getting hypertension (Chiang et al. 1996). Insertion of the I allele seems to be related to enhanced endurance performance, while deletion of the I allele seems to be associated with power- and strength performance (Nazarov et al. 2001; Qi et al. 2008).

The *ACE* I/D polymorphism is one of the over 160 polymorphisms at the *ACE* Locus . The physiological importance of the I/D polymorphism was discovered by its association with plasma *ACE* levels. There is a possibility for more functional polymorphisms. (Villard et al. 1996) Several investigators started studying the association between other variants in the gene and plasma levels, to find functional polymorphisms. Many of them are not in linkage disequilibrium with the *ACE* I/D polymorphism. (Sayed-Tabatabaei et al. 2006) 2 quantitative trait loci (QTLs), located on chromosome 4, are suggested to be functional polymorphissms. (Kammerer et al. 2004)

ACE inhibition can reduce the cross section of muscle fibers. (Morley, 2009, Sumukadas et al. 2006, Zoll et al. 2006) . Genetic inhibition of *ACE* can manifest in increased trainability of aerobic fitness while activities demanding a high power is negatively affected (Jones et al. 2002). It has been found in a large cohort study that genetic inhibition of *ACE* via the I allele in the *ACE* genotype can manifest in reduced trainability of aerobic fitness (Defoor et al. 2006) and that subjects with the *ACE* I show improved gains in maximal oxygen uptake with exercise rehabilitation (Danser et al. 2007; Defoor et al. 2006). Similarly and *ACE* inhibition blunts load-induced muscle growth in rats (Gordon et al. 2001).

This difference in increased trainability is possibly explained by modified transcript expression of pro-angiogenic factors, which reflects the mechanism that instructs the elevation in muscle capillarity post exercise (Schmutz et al. 2010). This transcript expression is lower in genotypes being homozygous (ACE-II) or heterozygous (ACE-ID) for the 'I-allele' (Vaughan et al. 2013). Capillary growth after exercise is reflected by elevated expression of a number of factors involved in the activation and stabilization of endothelial cells. In particular it is suggested that *Tenascin-C* mediates the effect of the vasoconstrictor AngII on growth of blood vessels because it is induced by AngII and is expressed at high levels in vascular structures in hypertension. Pharmacological inhibition of *ACE* blunts the muscle response of the pro-angiogenic factor *Tenascin-C* to endurance training in coronary artery disease patients (Zoll et al. 2006) Besides of *Tenascin-C*, hypoxia sensor *HIF-1α* is known to provokes in the muscle under hypoxia a specific gene expression response causing remodelling of existing blood vessels, a shift towards glycolytic metabolism and altered myogenic regulation in muscle as modified by the level of muscle

activation. The negative impact of *ACE* inhibition on endurance performance in man may be explained by the 'double' role of AngII, in vasoconstriction and capillary growth (angiogenesis). In resting muscle, the role of AngII is to restrict blood from entering non-active muscles in order to direct metabolic substrates to active tissues with enhanced energy turnover (Andersen and Saltin 1985). The AngII-mediated vasoconstriction is overridden with the onset of contraction through flow-induced dilatation of conduit arteries and arterioles and is expected to promote angiogenesis by activating the endothelial cell population that constitutes the capillary wall (Brothers et al. 2006; Hahn et al. 1995). Different studies support the notion that a switch in AngII action from arterioles to the endothelium of the perfused vessel lumen facilitates exercise-induced capillary growth in human skeletal muscle (Petersen et al. 2007; Staessen et al. 1987). Awareness of the existence of such a mechanism could develop major economic and clinical repercussions on current Health Care practice. Pharmacological inhibition of AngII action may have undesirable side effects, particularly, in the situation when one wants to exploit the sympatholytic and angiogenic benefits of physical therapy. This activity-dependent mechanism has rarely been valued in pharmacological studies of hypertension. An interaction between physical activity and angiotensin 2-mediated angiogenesis may also explain the large differences in therapeutic efficiency of current anti-hypertensive treatment.

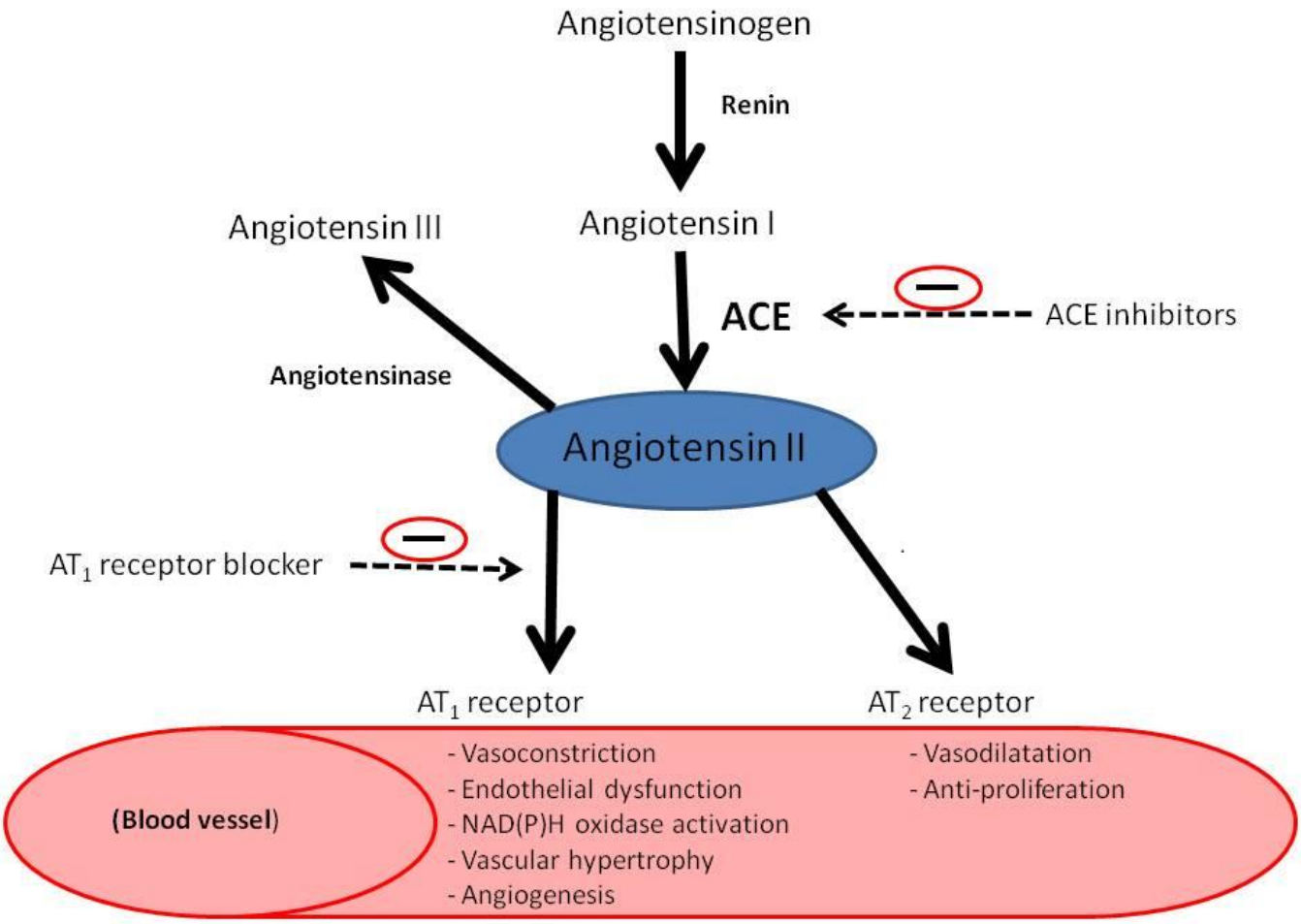

Figure 1: The renin angiotensin system
After angiotensinogen is produced by the liver it can via renin be converted into AngI. AngI is converted into AngII via ACE. Angiotensin II mainly works via AT_1 recepter and has the opposite function via the AT_2 receptor, but the AT_2 receptor is not so much present in the adult human body. AngII can be converted into AngIII via angiotensinase which has no primary function in the body. AngII signalling can be modulated by ACE inhibitors, and AT_1 receptor blockers.

Angiotensin II

AngII elevates arterial pressure as a result of its vasoconstrictor action and indirect by increasing the extracellular fluid volume by decreasing the excretion of salt and water of the kidney. AngII mediates its functions through two receptors: AngII type 1 receptor (AT1) and AngII type 2 receptor (AT2). Most of the recognized actions of AngII are mediated by AT1 receptors (Allen et al. 2000). In addition to the renal effects of increasing water and sodium retention, AT1 receptor activation evokes G-protein-dependent and –independent responses in the arterial vascular smooth muscle (Kimura et al. 2004). This includes vasoconstriction, activation of the pro-oxidant enzyme NAD(P)H oxidase (Lopez et al. 2003) and stimulation of vascular cell growth and proliferation (Wolf and Wenzell, 2004) and extracellular matrix formation (Otsuka et al. 1998). AT2 receptors are prevalent in fetal tissue, implicating a role in growth and development, and are expressed at much lower levels in adults. (Allen at al. 2000). Less is known about the regulation and action of the AT2 receptors. This could be the result of the limited distribution and density of AT2 in relation to AT1 and the antagonistic interaction those receptors could have. For instance, a few studies suggest that the effects of AT2 receptor activation promote vasodilatation and anti-proliferation (Siragy et al. 1999a; Siragy et al. 1999b).

AngII is degraded to angiotensin III by angiotensinase.

Angiotensin II and exercise

During exercise the renin-agiotensin system is stimulated and there is an increase in renin activity (Fasola et al. 1966; Kotchen et al. 1971, Fagard et al. 1985). Various studies show increases in AngII after a maximal incremental exercise test (Aldigier et al. 1993, Miura et al. 1994, Staessen et al. 1987, Braith et al. 1992, Kato et al. 1996, Danielsen et al. 1988, Fagard et al. 1985) but not all studies (Shim et al. 2008, Blanchet et al. 2005). AngII concentrations are also elevated after non maximal incremental or submaximal exercise test (Warren et al. 2001, Pedersen et al. 1986, Kinugawa et al. 1992; 1996 & 1997, Maher et al. 1975, Katzman et al. 1987) but not in the study of Braith et al. 2003. A critical note by these studies is that they show very high AngII values in serum. As example Staessen has 20 pg/ml at rest while natural values of the 'real' serum AngII levels are near 10pg/ml (Nussberger et al. 1986).

It should be noted that the majority of studies on *ACE* had a clinical focus and that AngII measurements were carried out only in a few situations. The main studies about AngII related to exercise are described in more detail below.

In the study of Staessen et al. (1987) subjects performed an incremental exercise test with 6 minutes of maintained external work load at 30% and 60% of their predetermined maximal exercise capacity. They found a positive correlation between plasma renin activity and AngII. Indicating that with increasing activity of plasma renin the plasma concentration of AngII augmented also. At 60% of the calculated maximal exercise capacity the slope of the regression line was steeper than at rest and at 30% of the calculated exercise capacity. This indicates that as the intensity of

exercise rise, the slope of the regression of plasma angtiotenisn II on renin activity become progressively steeper.

The study of Aldigier et al. (1993) also shows an increase in AngII with increasing intensity. In the study performed eight normative male subjects consecutive exercise bouts at 20%, 40%, 60% and 80% of their peak power output (PPO) on a cycle ergometer. Compared to the resting levels there was an increase in Angiotensin II of 1.50 (20% PPO), 1.48 (40% PPO), 3.18 (60% PPO) and 7.93 (80% PPO) times. Due to the relative long exercise steps, it should be noted that the increase in AngII is not likely to be related to the exercise duration but to the exercise intensity. What corresponds with the study of Staessen et al. (1987) is that there seems to be an exponential relationship between exercise intensity and AngII serum levels.

As seen above, exercise increases the AngII levels with the highest increases seen after high intensity exercise. It is not clear how elevations in AngII are related to the exercise intensity and exercise duration but it seems to be exponential related to external power (Aldiger et al. 1993, Staessen et al. 1987). These studies had been done with different exercise protocols. It is therefore unclear how serum angiotensin II levels depend on the intensity and time of the exercise.

We plotted data from different studies with submaximal exercise protocols into one figure (see figure 2). The studies had large differences in absolute angiotensin II values, so we normalized the values by rescaling the resting values to 1 and recalculated the other AngII levels. In the majority of studies the intensity level of the submaximal exercise was based on the persons VO2-max and in other studies on the subjects maximal workload or heart rate reserves.

Figure 2 shows that there seems to be a threshold at an intensity around 60% VO_2max/ 70% of the workload which evokes a sudden increase in AngII levels. This steep increase in AngII occurred during exercise above the anaerobic threshold.

2a)

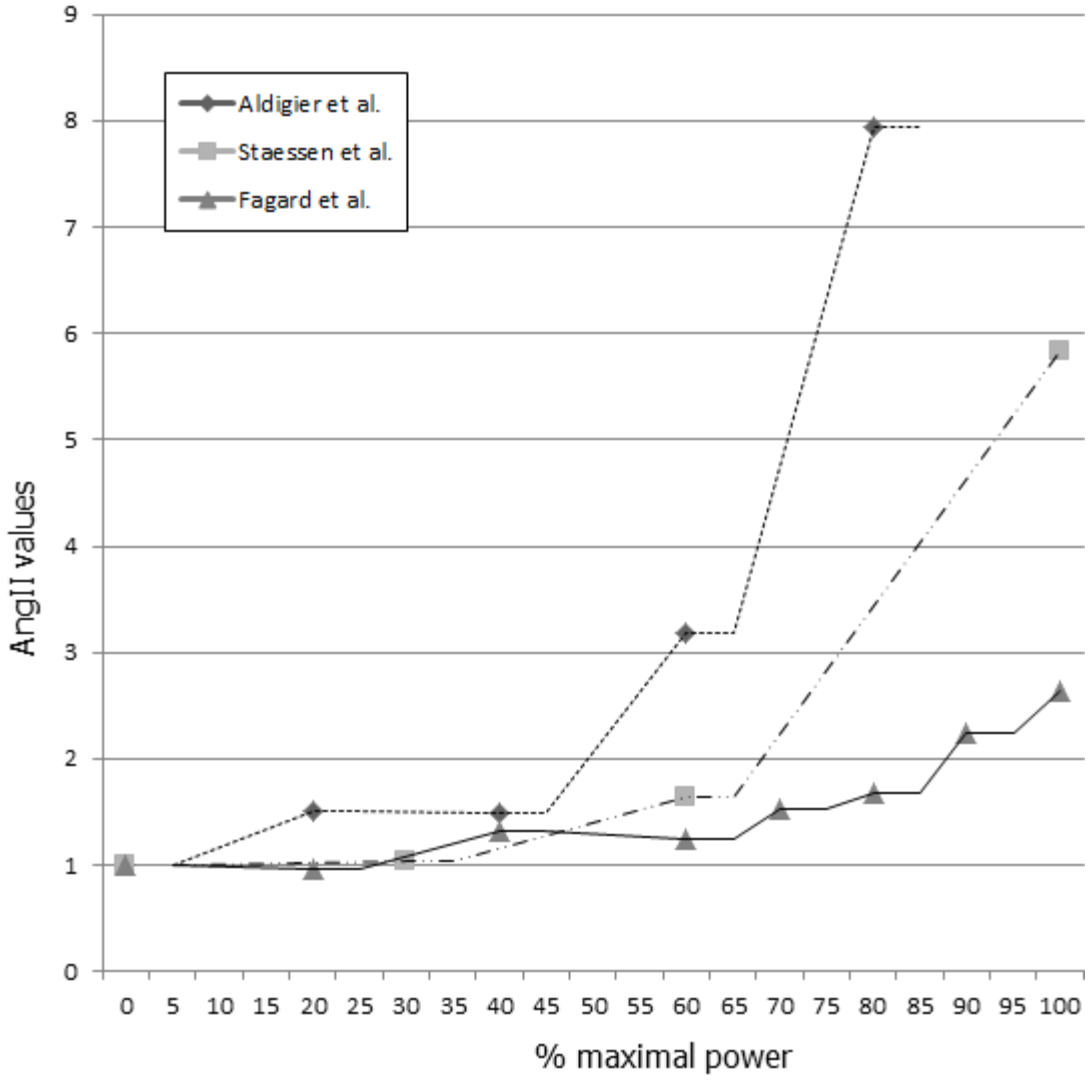

Figure 2a-c: Increase in AngII levels as seen from the resting levels in different studies. Studies with submaximal exercise protocols depending on %workload (a, above), %VO2-max (b, page 17) and heart rate reserve (c, page 18).Values are normalized values by rescaling the resting values to 1 by dividing the mean values of the pre levels by itself and dividing the other post AngII levels by the same number.

2b)

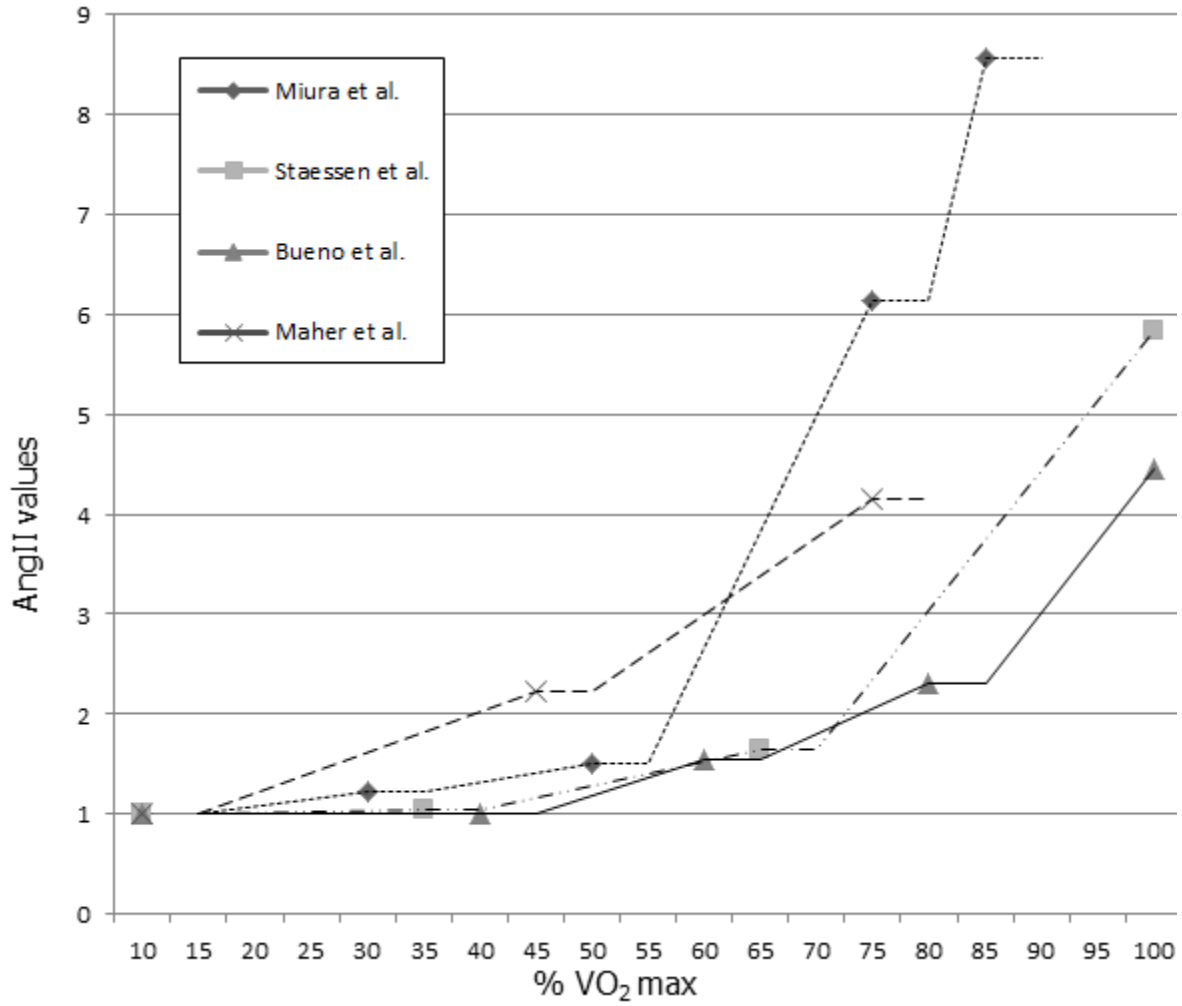

Figure 2b: *description of figure 2 see page 16*

2c)

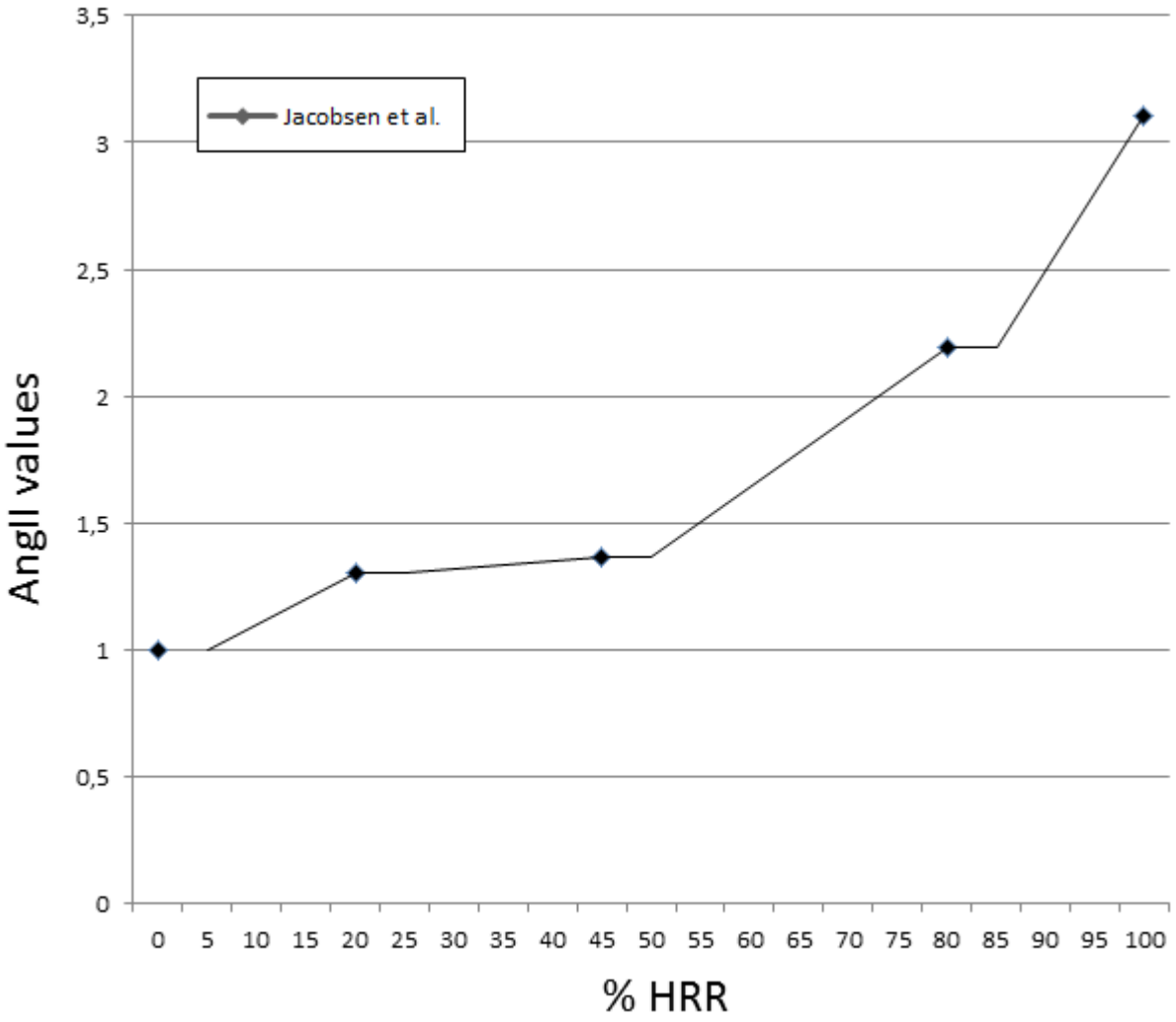

Figure 2c: *description of figure 2 see page 16*

An enhanced generation of angiotensin I might be considered to explain the steep increase in the plasma concentration of AngII during severe exercise. But according to Steasen et al. (1987), the capacity of the pulmonary vascular bed to convert AngI is very high comparing to the amount of the circulating decapeptide and is therefore not rate determining.

Another reason could be a decreased breakdown of AngII. In vivo, AngII is very rapidly removed from the bloodstream and degraded by most tissues including the vascular endothelium. In contrast, the study of Kosunen et al. (1976) shows that the AngII concentration could remain enhanced for hours after exercise. It has been demonstrated in the dog that up to 75% of AngII is removed in one circulation by the liver, head, kidneys, and hindquarters when singly perfused, whereas no AngII disappears across the pulmonary circulation. Four male athletes were investigated before and after running three times a 100m sprint with 5 minutes of rest in between. Direct after the last sprint there was a 4.2 fold increase in AngII levels compared to the resting levels and a 6.6 fold increase after 30 minutes of exercise and after 1 hour, 3 hours, and 6 hours of exercise there was respectively still a 3.7, 3.0, 1.7 times higher level of AngII.

An example for a potential mechanism of modulated AngII breakdown above the anaerobic threshold could be the induced acidosis evoked by exercise. With exercise at low intensity levels the pH hardly changes. However, at higher intensity exercise when the aerobic capacity is no longer sufficient, the anaerobic mechanisms are invoked and the pH can decline below 7.2 (Staessen et al. 1987). The angiotensinase activity, which breaks down AngII into angiotensin III, is pH

dependent. Within the pH range of 7.0 to 7.4 the activity can be reduced by 50% and can so inhibit the degradation of AngII thereby leading to an increase in AngII levels (Khairallah, 1967).

ACE genotype and exercise

Verry little is known what the effect is of the *ACE* genotype during and after exercise upon the AngII levels. In the study of Woods et al. (2003) they stated that there was no influence of genotype upon the exercise-related rise in *ACE* activity. Ang II levels were unrelated to *ACE* genotype and *ACE* activity. An important note is that the study only included six participants and the protocol of the cycling test was submaximal at 70% VO2-max.

ACE inhibition and exercise

Two studies investigated the effect of *ACE* inhibition in AngII levels during exercise. The studies of Aldigier et al. (1993) and Miura et al. (1994), as described above, had a cross-over design with a group of respectively 8 and 3 normotensive Caucasian male participants, receiving 50 mg of the *ACE* inhibitor captopril for three days. The subjects also received a dose in the morning of the exercise test. In both studies lower AngII levels were observed in the group who took the *ACE* inhibitor. Miura et al. (1994) found a treatment effect ($p=0.044$), but Aldigier et al. (1987) did not identify a treatment effect ($p=0.37$). In the study of Gavin et al. (1985) the effect of lowering *ACE* levels by captopril was investigated on angiogenic growth factor responses in the muscle to exercise in rats. Vascular endothelial growth factor

(*VEGF*) functions via binding to the *VEGF* receptors Flk-1 and Flt-1. They found that captopril did reduce Flk-1 mRNA 30–40%, independently of exercise but did not affect the rest or exercise levels of *VEGF* and Flt-1 mRNA. (Gavin et al. 1985)

Angiotensin II and Circadian rhythm

AngII shows a circadian rhythm in normal subjects whose acrophase is detectable in most studies early in the morning (Fersini et al. 1975, Cugini et al. 2004 Rittig et al. 2006). Only the study of Veglio et al. (1987) reports a low-amplitude circadian rhythm of which its acrophase is in the late afternoon. The angiotensin converting enzyme (ACE) shows a circadian rhythm as well with its circadian acrophase in the afternoon, suggesting a negative feed-back (Cugini et al. 2004). The daily variation of the renin-angiotensin-aldosterone systes is influenced by many factors. Physical activity and rest affect both the mesor amplitude and acrophase (Cugini et al. 1978). Moreover, circadian rhythm seems to be influenced by the race. The circadian rhythm is absent in people with hypertension (Fersini et al. 1975, Cugini et al. 2004)

Aims and thesis outline

The aim of this research was to expose the pathway by which AngII is implicated in exercise-induced capillary growth of human muscle. We hypothesize that the blunting of AngII production with concomitant exercise reduces improvements in metabolic fitness by removing an important stimulus for capillary growth in exercised muscle.

In **Chapter 2** it is described how serum AngII levels are increased after exercise and depend on exercise time and exercise intensity, and *ACE* I/D gene polymorphism related capillary perfusion. **Chapter 3** presents a study that exposes the role of AngII in angiogenic gene expression in exercised muscle by assessing the effect of *ACE* inhibition by medication with in **chapter 4** from the same study the association of *Tenascin-C* with ACE-modulated muscle angiogenesis after endurance training. As adjustments to endurance exercise and elevated altitude have been shown to be affected by the *ACE* I/D gene polymorphism we assessed in a training study in **Chapter 5** whether the response of the pathway that sets aerobic capacity would be modified by the *ACE* I/D gene polymorphism. The thesis is concluded with a general discussion in **Chapter 6** of the implications of the results for our understanding of the relevance of AngII and *ACE* I/D gene polymorphism in the human body and the implication of inhibited AngII production during exercise.

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Chapter 2

EXERCISE INTENSITY MODULATES CAPILLARY PERFUSION IN CORRESPONDENCE WITH *ACE* I/D MODULATED SERUM ANGIOTENSIN II LEVELS

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Abstract

During exercise the renin-angiotensin system is stimulated. We hypothesized that the increase in serum angiotensin II (AngII) levels after exercise is dependent on exercise intensity and duration and secondly that people with the ACE-II genotype will show a higher increase in AngII serum levels. We also assumed that perfusion of upper limbs is transiently reduced with maximal cycling exercise and that subjects with the ACE-II compared to the ACE-ID/DD genotype will have a higher capillary perfusion due to lower AngII levels. Ten healthy subjects completed a maximal exercise test, a 12-minutes exercise test at ventilatory threshold and a 3-minutes test at the respiratory compensation point. AngII serum levels and capillary recruitment of the skin in the third finger was measured before and after exercise and breath-by-breath gas exchange during exercise was assessed. Baseline levels of AngII levels were lower prior to the 3-minutes test which took place on average 5 days after the last exercise. A two-fold increase compared to baseline levels was found for AngII only immediately after the 3-minutes test and not after the maximal exercise test and 12-minutes of exercise. Subjects without the I allele showed a decrease in AngII values after the maximal test in contrast to subjects with the ACE-II/ID genotype. Subjects with the ACE-II genotype had a 1.8 times significant higher capillary perfusion in the finger after exercise. A trend was observed for a 34.3% decreased capillary recruitment in the ACE-ID/DD genotype after exercise. We conclude that the rise in AngII after exercise is intensity dependent and that variability in serum AngII and capillary perfusion is related to the *ACE* I/D polymorphism.

Introduction

Angiotensin II (AngII) elevates arterial pressure because of its vasoconstrictor action and restricts blood from entering non-active muscles thereby directing metabolic substrates to active tissues with enhanced energy turnover (Andersen and Saltin 1985). This mechanism helps to redirect blood to active muscles which work depends on the substrate supply (Secher et al. 1977). The AngII-mediated vasoconstriction is overridden with the onset of muscle contraction through flow-induced dilatation of conduit arteries and arterioles and promotes angiogenesis by activating the endothelial cell population that constitutes the capillary wall (Brothers et al. 2006; Hahn et al. 1995). Different studies support the notion that a switch in AngII action from arterioles to the endothelium of the perfused vessel lumen facilitates exercise-induced capillary growth in human skeletal muscle (Petersen and Greene 2007; Staessen et al. 1987). There is evidence that the role of AngII in stimulating angiogenesis relies on capillary perfusion (Petersen and Greene 2009).

During cycling exercise, the renin-angiotensin system is stimulated (Staessen et al. 1987). Different studies show an increase in AngII after high intensity exercise, indicating that organs which cannot overcome AngII mediated vasoconstriction are decreasingly perfused (Fagard et al. 1985; Kosunen and Pakarinen 1976; Staessen et al. 1987; Aldigier et al. 1993). Limited studies investigated AngII levels related to exercise intensity and duration, but it seems that there is an increase in AngII at an intensity above the anaerobic threshold (Maher et al. 1975, Staessen et al. 1987, Miura et al. 1994).

Human subjects carrying the D-allele of the *ACE* I/D gene polymorphisms (i.e. ACE-ID and ACE-DD genotypes) have respectively approximately 30% and 60% higher serum and tissue *ACE* activity and more serum AngI is converted via *ACE* to AngII than in subjects with the ACE-II genotype. However, only less than 20% of the variability in circulating *ACE* activity can be explained by the *ACE* I/D polymorphism (Danser et al. 2007). Subjects with homozygous (ACE-II) or heterozygous (ACE-ID) for the 'I-allele' show reduced capillarity at rest but amplified mitochondrial biogenesis after bicycle-type endurance exercise and training, suggesting a critical role of ACE-modulated vascular tone in the regulation of the response to exercise (Defoor et al. 2006; Vaughan et al. 2013).

We aimed to detect changes in human AngII levels after exercises at different intensities. We hypothesized that the increase in serum AngII after exercise is dependent on the exercise intensity and the exercise duration. We also hypothesized that people with the ACE-II genotype will show a higher increase in AngII serum levels after cycling exercise due to their lower AngII resting values (Vaughan et al. 2013). In order to establish the suggested association between serum AngII and capillary perfusion with exercise (Petersen and Greene 2007), we tested whether serum AngII levels relate to exercise intensity and capillary perfusion in skin, in a body compartment being accessible during exercise, and whether this depends on the *ACE* I/D genotype. We assumed that perfusion of the finger is reduced with maximal cycling exercise and that subjects with the ACE-II genotype will have a higher capillary perfusion as they have less increased capillary density and a genetically-reduced potential for vasoconstriction (Vaughan et al. 2013; Brothers et al. 2006). Consequently, the relative change in perfusion and exercise-induced Ang-

II levels in serum will be less pronounced than in D-genotypes for which vasoconstriction is overridden to a larger extent.

Methods

On three separate occasions, ten healthy young subjects performed three exercise tests on three separate occasions at the same time of the day on an electronically braked cycle ergometer (Excalibur Sport, Corval Lode B.V., Lode Medical Technology, Groningen, The Netherlands) in an air-conditioned room, where the temperature was kept between 15 and 20 degrees. All subjects gave written informed consent before participating in the study and participation was accepted according to the anamnesis forms. Before the first exercise test the subjects height and weight were measured. Mucosal cells were removed from inside of the cheeks by simply twirling/rubbing an ear bud against the inner cheek wall. During all tests the same personalized bicycle settings were used and the subjects were instructed to keep their pedal frequency constant at 80 rotations per minute (rpm), which was displayed on an analog display on the handlebar. Subjects were asked to rate their perceived exertion (RPE) on a 20 points Borg RPE scale every minute. 2ml of blood was collected from the upper arm via the median cubital vein before cycling and 3, 6, 9 and 12 minutes after the cycling tests. Blood collection was done by a professional person via an intravenous cannula from the upper arm, while the subjects were in a sitting position. During the cycling exercise the subjects' VO_2 (oxygen uptake) and VCO_2 (CO_2 production) were measured breath by breath via expired air using a gas analyzing system (Cosmed Quark, Cosmed S. R. L., Rome,

Italy) and the subjects' heart rate was measured with a Polar heart rate belt. From these data the maximal oxygen uptake (VO_{2peak}) and the respiratory exchange ratio (RER) was established.

At the first occasion the subjects performed a maximal incremental exercise test (maximal test). Subjects started cycling for three minutes at 50 Watt and the resistance was increased every minute by 25 watt until exhaustion. The test ended when the pedal frequency dropped below 65 rpm. After the first test the ventilatory threshold (VT) and respiratory compensation point were identified using the V-slope and ventilatory equivalent methods (Sue et al. 1988). To investigate the effect of exercise duration and intensity on AngII levels at and above the AT, subjects performed two other exercise tests at different occasions on the same time of the day with at least one day of rest in between. At the second occasion subjects cycled after a 3 minutes warm up of 50 Watt for 3 minutes at the intensity where they reached their RCP during the maximal test. At the third occasion subjects cycled for 12 minutes at an intensity at which they reached their VT the maximal test. When the RPE of the subject during the tests dropped below 11 or was above 13, the external power was adjusted with 25 Watt.

Capillary recruitment

To assess perfusion in a non-active body compartment we used the capillary microscope which can non-invasively and directly visualize the perfusion of the skin in the finger. Absolute and relative capillary recruitment was measured before and directly after the maximal test as described previously (Serne et al. 1999). Briefly, two separate visual fields of 1 mm^2 were recorded 1.5 mm proximal to the terminal

row of capillaries in the middle of the nail fold of the third finger. A characteristic capillary (i.e., a capillary that was constantly perfused and had an eye-catching morphological feature) was kept on the same spot exactly in the center of the visual field (marked by a dot on the monitor) to ensure that capillary density was measured in the exact same visual field during the entire experiment. Capillary density was counted as the number of continuously erythrocyte-perfused capillaries per square millimeter (n/mm²) during 15 seconds and after 4 minutes of arterial occlusion. Capillary recruitment was calculated as the relative increase in capillary density from baseline to capillary density after the 4 minutes of arterial occlusion. The procedure was repeated using a visual field adjacent to the first visual field and data concerning capillary densities are the mean of two measurements. The number of capillaries was counted off-line by a single experienced study physician (Jorn Woerdeman).

Assessment of angiotensin II levels

Blood (2 ml) was withdrawn from the venous cannula and put into vacutainers containing 60 μl AngII inhibitor cocktail, comprising 13.35 μl of O-Phenanthroline and Pepstatine A in DMSO mixed with 46.65 μl of EDTA and PHMB in aqueous solution (SPI bio, Bertin pharma, Versailles, France). The samples were immediately centrifuged at 10,000 rpm (3,000 g), at 4^{o}C for 12 min. The supernatants were separated, snap frozen in liquid nitrogen and stored at -80^{o}C, transferred with dry ice to MMU Manchester and stored at -80^{o}C until analysed. Plasma was eluted with C18 phenyl cartridge which were conditioned with 2 ml of methanol and then rinsed with 2 ml of water. 0.9 ml of cold plasma was rapidly passed through the cartridge

and subsequently washed with 1 ml of water. Absorbed angiotensins were eluted with 1 ml of methanol into conical polypropylene tubes. The eluate was evaporated to dryness by means of a nitrogen gas stream at room temperature and the residue was stored at -20^{o}C.

We have characterized the performance of the AngII assay in pilot experiments where we spiked AngII in the sample before the measurements (data not shown). These experiments show that the assay recovers 85% of the AngII peptide in blood plasma. As well we find that the detection of the AngII signal is linear for the range between 2 and 100 pg/ml.

AngII was assessed with a commercially available AngII enzyme immunoassay kit (SpiBio, Montigny Le Bretonneux, France) according to the manufacturer's instructions. The characterization of this kit performance has been published (Volland et al. 1999). Briefly, the plasma samples were incubated with 100 μl of EIA, 50 μl, 100 μl of borane-trimethylamine and with 100 μl of anti-AngII IgG tracer. Samples were incubated with 200 μl of Ellman's reagent. The plate was red with a single quick read on an Absorbance Microplate Reader (ELx800, BIO-TEK) with wavelength 405 after 30 minutes, 1 hour and 2 hours of incubation.

ACE Genotyping

DNA was extracted from mucosal samples using a custom designed protocol. The mucosal mouth swabs were extracted with 800 μl of methanol with vortexing and left to evaporate in air under a stream of nitrogen gas. The pellet was resuspended in 100 μl of sterile water and stored at -20^{o}C.

ACE genotyping was carried out with Polymerase chain reaction (PCR) as described by Evans et al. (1994). PCR reactions were run with a mix of the three primers using Sybr Green master mix (Applied Biosystems) on a Applied Biosystems Real Time PCR system (SepOnePlus, Life Technologies). This involved 45 standard cycles of denaturing at 95ºC for 15 s followed by annealing and extension at 55ºC for 1 min. Amplicon identification followed using a melting curve analysis between a temperature range of 70ºC to 80ºC. The identity of the amplified sequence for the ACE-I and ACE-D genotype was validated by sequencing of the PCR products with the specific primers (Microsynth, Balgach, Switzerland). The presence of the short amplicon for the I-allele was identified by a lower melting temperature (73.5^{o}C; 72.5 - 74^{o}C) compared to the longer D-allele (75.5^{o}C; 74.5 - 76.5^{o}C) respectively.

Statistics

Results are mean ± SEM. For AngII the data was logarithmic transformed and an analysis of variance (ANOVA) with repeated measures was used to compare time and exercise intensity overall and between the *ACE* genotypes. To compare the effect of exercise among *ACE* genotypes, AngII levels were background-corrected and related to the mean values of the respective genotype before exercise. For capillary parameters, capillary recruitment and percentage capillary recruitment were compared by a Student's paired t-test.

Results

Subject (n=10) characteristics at baseline and during the three tests are reported in table 1a and 1b. Four subjects carried the ACE-II allele, and six the ACE-ID/DD allele.

Blood samples data are shown in figure 1 and 2. No correlation was found between the blood lactate levels and the serum AngII levels. All indices of metabolic strain, i.e. VO_{2peak}, RER, heart rate and lactate levels after exercise were elevated after all the three exercise tests and remained high over the study period (figure 1, table 1). The highest increases in indices of metabolic strain were observed after the maximal test.

There was a difference in AngII levels before the exercise. AngII levels before the first tests at maximal intensity were higher than after the tests done in the course of the next days. AngII levels were significantly increased after the 3-minutes exercise test (figure 2) but not after the 12-minutes and maximal test. A main effect of the presence of the I-allele was identified for the course of changes in AngII levels relative to baseline after exercise in the maximal test ($p=0.041$), but not the 3-minutes ($p=0.357$) and 12-minutes test condition ($p=0.395$). Subjects with the II genotype showed a maintained level of AngII whereas AngII levels decreased in subjects lacking the I allele after the maximal test (figure 3).

Table 2 shows a non-significant increase in capillary recruitment in subjects carrying the *ACE* II-allele while a trend for a decreased capillary recruitment after exercise was observed in subjects carrying the ACE-ID/DD allele. When we compared the genotypes we observed that capillary recruitment after exercise was significantly less in the ACE-ID/DD group compared to the *ACE* II-allele. No indication for an effect for capillary recruitment post exercise was seen in function of the I-allele (data not shown).

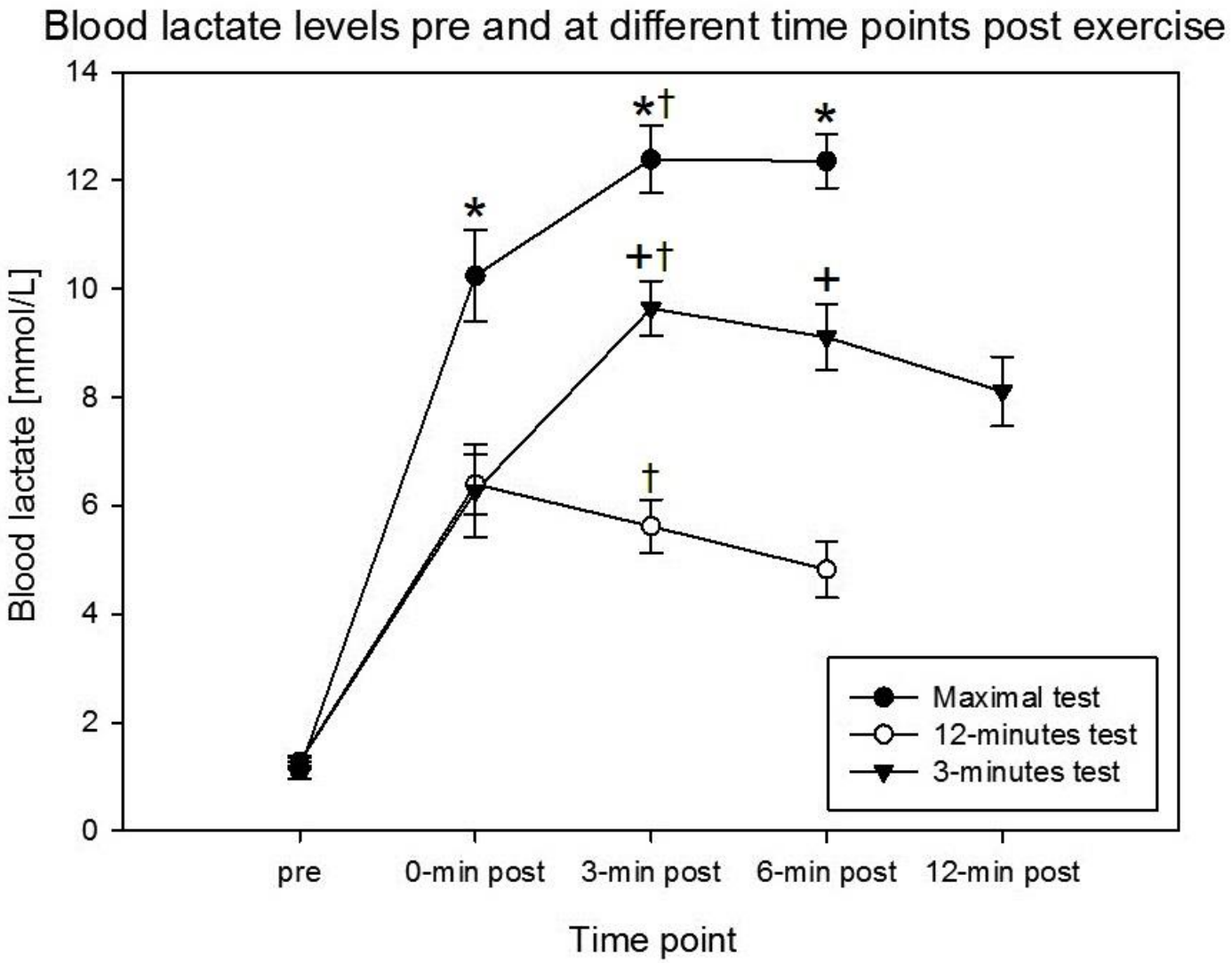

Figure 1: *Metabolic strain of the three exercise conditions* Mean + SEM of serum lactate levels at the measured time points post exercise. * significantly different from 12-minutes test and 3-minutes test ($p<0.01$), + significantly different from maximal test and 3-minutes test ($p<0.01$), † significantly different from pre , 0-minutes post and (if measured) 9-minutes post ($p<0.05$). (repeated measures ANOVA, n=10).

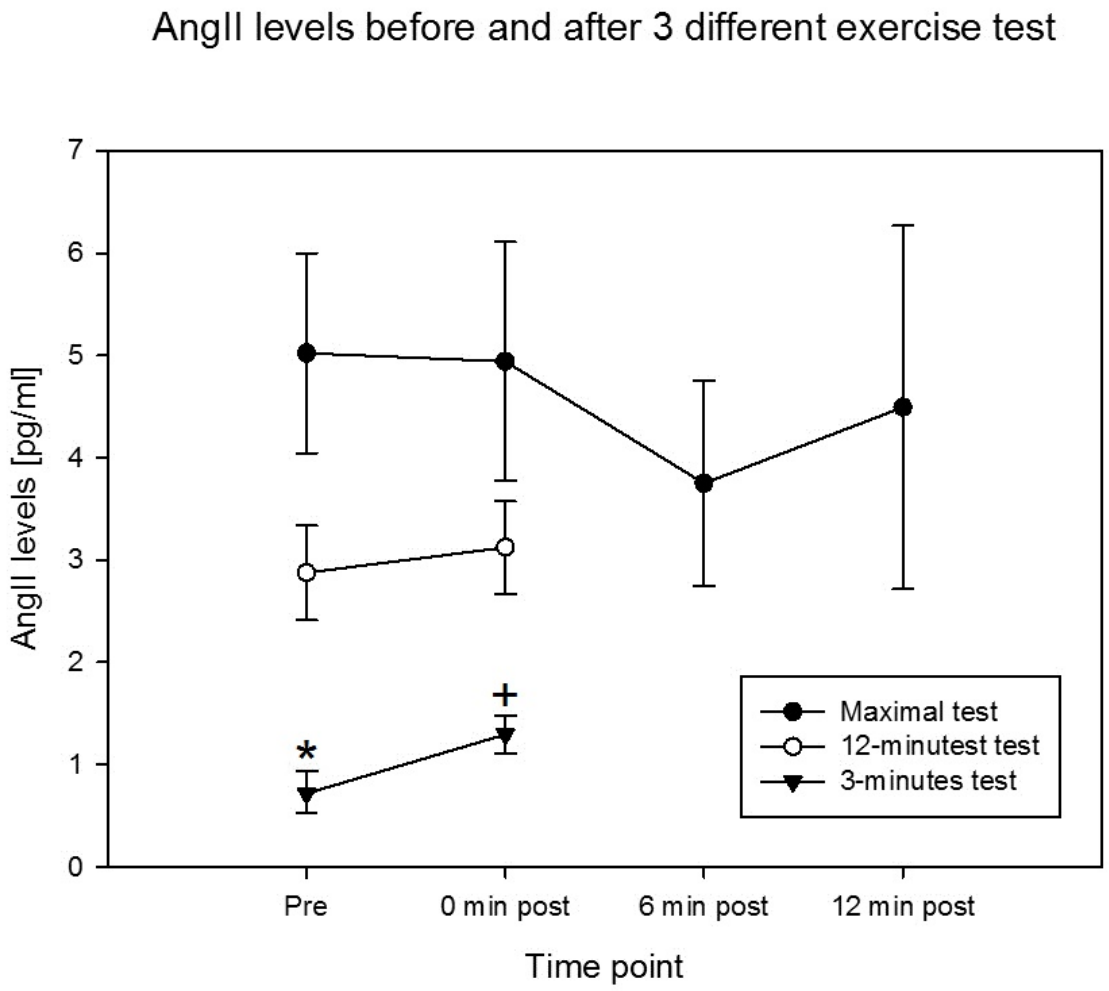

Figure 2: *Serum AngII levels after exercise.*

Mean + SEM of AngII concentration in capillary blood (serum) at the measured time points post exercise. * significantly different from 12-minutes test and maximal test ($p<0.05$) and + indicates significantly different from 12-minutes test, maximal test and 3-minutes post ($p<0.05$); (repeated measures ANOVA, n=10).

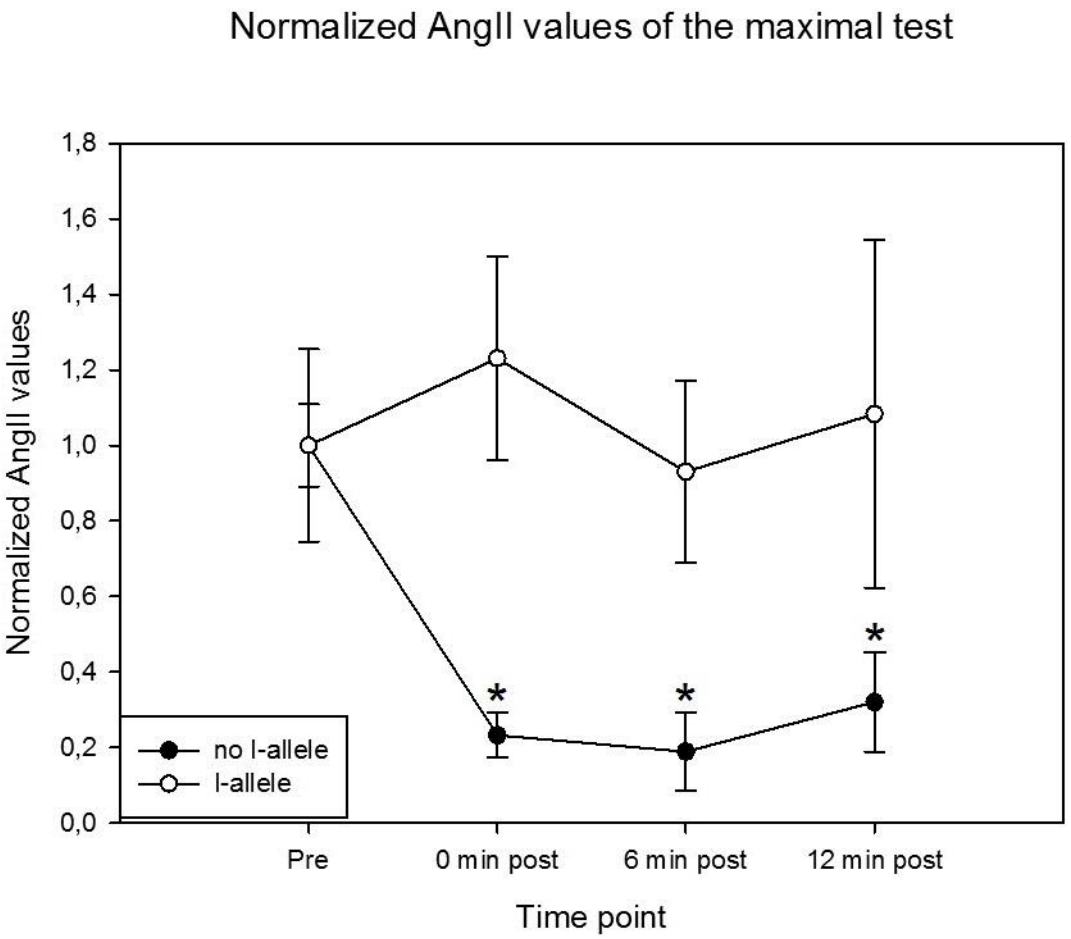

Figure 3: *ACE I/D dependence of AngII levels after the maximal exercise test*

Mean + SEM of AngII concentration in capillary blood serum for carriers and non-carriers of the *ACE* I-allele. * indicates significant different from the I-allele and from no I-allele (pre) $p<0.05$ (repeated measures ANOVA, n=10)

Table 1a: Baseline characteristics of the studied subjects.

Age (year)	29.6 ± 2.1
Length (cm)	180.9 ± 7.6
Mass (kg)	76.1 ± 5.6
Resting heart rate (beats/min)	65.8 ± 9.0

Table 1b: Physiological characteristics of the studied subjects during the three exercise tests.

		Power (watt)	**Heart rate** (beats/min)	**VO_2** ($ml\ O_2/min$)	**VO_2/kg** ($ml\ O_2/kg/min$)	**RER**	**RPE** (6-20)	**Heart rate before exercise** (beats/min)	**Time after last exercise test** (days)
Peak (last 30 sec)		353 ± 49	187.4 ± 7.4	3885.2 ± 508.9	51.0 ± 4.8	1.32 ± 0.07		70.8 ± 3.0	
Maximal test	*AT*	215 ± 54	143.9 ± 12.6	2701.8 ± 604.9	35.4 ± 6.5				
RCP		301 ± 51	161.3 ± 8.3	3387 ± 482.7	44.24 ± 4.6				
12 min test (last 30 sec)		230 ± 48	157.4 ± 5.2	3173.1 ± 527.4	41.6 ± 5.3	1.04 ± 0.03	14.3 ± 0.9	73.8 ± 3.5	5.0 ± 0.9 (range: 2-9)
3 min test (last 30 sec)		310 ± 42	171.7 ± 9.0	3589.6 ± 458.3	47.14 ± 4.5	1.22 ± 0.07	16.6 ± 0.8	69.9 ± 4.0	8.0 ± 1.5 (range: 2-15)

VO_2 =oxygen uptake, RER = respiratory exchange ratio, RPE = rate of perceived exertion

Table 2: Capillary recruitment in numbers and percentages before and after the maximal test.

Genotype	Test	Pre	Post	p
II	Capillary recruitment (%)	35.5 ± 5.5	43.3 ± 4.6	*0.73*
	Capillary recruitment	18.3 ± 2.6	26.8 ± 2.2	*0.11*
ID/DD	Capillary recruitment (%)	36.2 ± 6.3	23.8 ± 4.1	*0.08*
	Capillary recruitment	15.9 ± 2.7	12.9 ± 2.4†	*0.19*

Values are means ± SEM. † Significantly different from capillary recruitment II at 0 min post ($p<0.05$)

Discussion

We aimed to detect changes in human AngII levels after exercise at different intensities.

The measured Ang II levels are in line with the values in the seminal report on how to measure Ang II in plasma and which identifies that the true levels in serum are close to 7-10 pg/ml (Nussberger et al. 1986). Overall it was observed that highest increases in indices of metabolic strain were observed after the maximal test. We hypothesized that the rise in AngII is dependent on exercise intensity and duration. A two fold increase in serum AngII concentration after intense exercise was found after the 3-minutes exercise test. This is comparable to the increase found in the study of Fagar et al. (1985). No significant increases in AngII were found after the 12-minutes test and maximal test with also no significant differences in AngII levels between the different time points after exercise. (Fig. 2).

As hypothesized the AngII values after the maximal exercise test were higher in the subjects carrying the ACE-II genotype. Subjects without the I allele showed a decrease in AngII values after the maximal test where people with the ACE-II genotype did not (Fig. 3). A main effect of the presence of the I-allele was identified for the course of changes in AngII levels relative to baseline after exercise under the maximal test, but not the 3-minutes and 12-minutes test condition. This might support a role of the duration of exercise for affecting serum AngII. We therefore conclude that the rise in AngII post exercise might be duration dependent and seems to be intensity and *ACE* genotype dependent.

It was further observed that as hypothesized subjects with the ACE-II genotype had a significant higher capillary perfusion in the finger after exercise compared to the ACE-ID/DD genotype. With a trend for a lower capillary perfusion in the ACE-ID/DD genotype post exercise, these data indicate that this body compartment is less perfused due to AngII dependent vasoconstriction during exercise. This mechanism is comparable with the study of Santana et al. (2011) in which they found a main effect in systolic blood pressure between the *ACE* genotypes after a maximal test and a cycling test at 90% AT. They also showed a protective effect on post-exercise diastolic blood pressure and MAP. The initial vasoconstriction may be overridden by maximal cycling exercise due to the systemic action of released nitric oxide (NO) and adenosine (Santana et al. 2011). We therefore conclude that there seems to be a relationship between AngII levels and *ACE* genotype and between *ACE* genotype and blood flow.

It was astonishing that a difference was noted for AngII levels at rest before each of the three tests (Fig. 2). We note that the values measured for AngII (see Fig. 2) are in the range of reported values (i.e. 2.3-18.6 pmol l-1; Ueda et al. 1998). It has been reported that exercise lowers hypertension and that this effect lasts certainly 13 hours (Kenney and Seals 1993). Santana et al. (2011) showed that only people with the I allele of the *ACE* genotype had post exercise hypotension and increased NO-release. As AngII levels in serum relate to blood pressure (Kaplan and Silah (1964) and the 3-minutes tests were by some subjects conducted 2 days after the maximal test, including subjects bearing the I allele the hypothesis is raised that the lowered AngII may be related to post exercise hypotension that lasts at least 15

hours (Kenney and Seals 1993). The heart rate before the 3 minutes test was non-significant higher, compared to the maximal test (table 1b).

Remarkable is also the variation in AngII levels between the subjects that can be revealed from the standard error (Fig. 2). Subjects with the ACE-II genotype had significant higher AngII values at all the time points after the maximal test then people without the I allele, who demonstrate decreased AngII values after the maximal test (Fig. 3). On the identified effect of *ACE* genotype on the AngII levels after the maximal test, we think the *ACE* genotype exerts a major influence on the standard error. These results indicate that the D-allele restricts production of AngII after exercise, while people without a D-allele maintain AngII levels after exercise.

A limitation of our study was that capillary recruitment measurements were only performed at the maximal test. Due to the short duration, we expect no exercise induced vasodilatation based on literature after the 3 minutes cycling test (Tschakovsky et al. 2002). Therefore we cannot rule out that for instance the increase in serum AngII immediately after the 3-minuts test is an acute phenomenon of vasoconstriction in non-exercised body parts that also took place in subjects exercising for longer duration and which did not halt exercise to allow sampling of this time point.

The increase in AngII levels after exercise causes the blood pressure to decrease but on the other hand it is expected to promote angiogenesis in the activated muscles by activating the endothelial cell population that constitutes the capillary wall (Brothers et al. 2006). A trend was observed that subjects with the ACE-DD/ID

polymorphism showed a decrease in capillary recruitment after exercise. Subjects with the ACE-II polymorphism had a significant larger recruitment of the relative capillaries after exercise compared to subjects with the ACE-ID/DD polymorphism.
The rise in serum AngII with the 3-minutes exercise protocol at the RCP highlights a role of high intensity exercise for a short duration. Taking into account that the maximal test also included a similar level of intensity we analyzed the role of exercise time of exercise-induced systemic vasodilatation. The identified decrease in capillary recruitment in the finger of ACE-ID-DD genotypes after the maximal test supports the contention of an elevated potential for vasoconstriction in non-exercised body parts in subjects which do carry the D-allele. In this regard the documented increase in *ACE* transcript expression in vastus lateralis muscle being recruited during bicycle exercise in ACE-DD genotypes (Vaughan et al. 2013) suggests a role of the *ACE* I/D polymorphism in controlling capillary recruitment with exercise via effects on *ACE* gene expression.

The data indicate that variability in the response of the major vasoconstrictor, AngII, in serum after exercise is *ACE* genotype-dependent and not related to blood lactate levels. In some contradiction to Danser et al. (2007) we see that in the controlled situation of maximal exercise, there is evidence for a role of the *ACE* I/D polymorphism in explaining variability in serum AngII and capillary perfusion. Thus, physical activity must be considered as an important confounder of previous conclusions. Awareness of the differences in AngII generation after exercise could have clinical repercussions on current Health Care practice. It may be one of the reasons why people with the ACE-II genotype and/or people taking *ACE* inhibitors show lowered gains as a result of exercise rehabilitation (Defoor et al. 2006).

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Author contributions

Author contributions: Sv.G., Ad.H, Jd.K., and M.F. conception and design of research; Sv.G. and J.W. (capillary recruitement measurements) performed experiments; Sv.G., J.W., and M.F. analyzed data; Sv.G. and M.F. interpreted results of experiments; Ad.H. and M.F. funding; P.V. and M.F. prepared figures; Sv.G. and M.F. drafted the manuscript; Sv.G., Ad.H., J.W., L.V., E.S. and M.F. edited and revised the manuscript

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Chapter 3

Inhibition of *ACE* alters the induction of pro-angiogenic and mitochondrial gene transcripts after a single exercise bout

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Abstract

Skeletal muscle responds to endurance exercise with a improvement of biochemical pathways that support substrate supply and oxygen dependent metabolism. This is reflected by enhanced expression of associated factors post exercise and appears to be specifically modulated by tissue perfusion and oxygenation. We hypothesized that transcript expression of pro-angiogenic factors (*VEGF*, *Tenascin-C*, *Angpt1*,*Angpt1R*) and oxygen metabolism (*COX4I*, *COX4I2*, *HIF-1*α) in human muscle after an endurance stimulus depends on vasoconstriction, and would be modulated through inhibition of *ACE* by intake of lisinopril.

Fourteen non-specifically trained, male Caucasians subjects, carried out a single bout of standardized one-legged bicycle exercise. Seven of the participants consumed lisinopril in the three days before exercise. Biopsies were collected pre- and three hours post-exercise from the *m. vastus lateralis*. *COX4I*1 (p=0.03) and *HIF-1*α (p=0.05) and *COX4I2* (p=0.04) mRNA levels showed an exercise-induced increase in the group not consuming the *ACE* inhibitor. Conversely, there was a specific exercise-induced increase in transcript levels for the pro-angiogenic factor *VEGF* (p=0.04) and a trend for increased *Tenascin-C* transcript levels (p=0.09) for subjects consuming lisinopril. The observations indicate that regulation of exercise-induced transcript expression of genes involved in angiogenesis and mitochondrial energy metabolism are to some extent regulated via an ACE-dependent mechanism.

List of abbreviations

ACE(i): Angiotensin converting enzyme (inhibitor)

AngII: Angiotensin II

Angpt1(R): Angiopoietin 1 (receptor)

ATP : Adenosine triphosphate

*COX4I*1: Isoform 1 for subunit 4 of cytochrome *c* oxidase

COX4I2: Isoform 2 for subunit 4 of cytochrome *c* oxidase

(c)DNA: (Complementary) deoxyribonucleic acid

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediaminetetraacetic acid

EIA: enzyme immunoassay

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HCL: Hydrochloric acid

HIF-1(α): hypoxia-inducible factor (α-subunit)

PCR: Polymerase chain reaction

(m)RNA: (Messenger) ribonucleic acid

rpm: revolutions per minute

VEGF: Vascular endothelial growth factor

VO_2-peak: maximal oxygen uptake

Introduction

Exercise causes intensity-dependent vasodilation and increases blood flow to contracting muscles (Andersen and Saltin 1985), ensuring an adequate oxygen supply to the contracting muscle. This exercise hyperaemia is challenged by activation of sympatho-adrenergic and nonadrenergic vasoconstrictor systems. After mild and moderate intense exercise there is an exercise-induced inhibition of AngII-induced vasoconstriction indicating that muscle contractions attenuate adrenergic and non-adrenergic vasoconstriction. During severe exercise there is a higher sympathetic activity and an increase in the activity of plasma rennin and a subsequent increase in the production of *ACE* and vasoconstrictor angiotensin II (AngII) is observed (Staessen et al. 1987). During increasing intensities of exercise the concomitant increasing activity in sympathetic vasocontrictor nerves is driven by a combination of central nervous coactivation of motor control and sympathetic outflow and muscle-derived afferents (Victor et al. 1995).

There is evidence that the role of AngII in stimulating angiogenesis relies on capillary perfusion of skeletal muscle (Petersen et al. 2007). AngII elevates arterial pressure because of its vasoconstrictor action and restricts blood from entering non-active muscles in order to direct blood flow to active tissues with enhanced energy turnover (Andersen et al. 1985). The AngII-mediated vasoconstriction is overridden with the onset of contraction through flow-mediated dilatation of conduit arteries and arterioles and promotes angiogenesis by activating the endothelial cell population that constitutes the capillary wall (Brothers et al. 2006; Hahn et al. 1995).

Training increases the capacity for aerobic ATP production in skeletal muscle through an elevation in the volume density of mitochondria in untrained subjects (Hoppeler et al. 1985). After cycling exercise under lowered ambient oxygen (hypoxia) there is a larger increase in the volume density of subsarcolemmal mitochondria compared to those residing between myofibrils, indicating that local adaptations are specifically modified by the amount of oxygen in the muscle during exercise (Desplanches et al. 1993; Schmutz et al. 2010; Vogt et al. 2001). As a result there are improvements in maximal oxygen uptake (VO_2-peak), aerobic power, fatigue resistance and also in subsarcolemmal mitochondria after trainingin hypoxia compared to normoxia (Hoppeler et al. 1992; Ponsot et al. 2006; Zoll et al. 2006*;* Flück et al. 2006).

Metabolic and vascular adaptations that may be involved in the hypoxia-specific improvement of muscle specific aerobic capacity after repeated endurance workouts include the enzymes operating at critical biochemical steps of mitochondrial respiration under lowered muscle oxygenation during severe exercise in hypoxia (Flueck et al. 2009; Richardson et al. 1995). One of the key metabolic enzymes is cytochrome *c* oxidase, which is the last enzyme in the respiratory electron transport chain converting molecular oxygen to water. After one bout of intense bicycle exercise under hypoxia isoforms 1 and 2 of oxygen-sensitive cytochrome *c* oxidase (*COX4I*1, *COX4I2*) plays a role in the enhancements of the aerobic capacity (Flueck et al. 2009; Richardson et al. 1995). After exhaustive endurance exercise in hypoxia the expression of *COX4I2* is elevated more than the isoform *COX4I*1 (Fukuda et al. 2007). A correlation between elevated *COX4I2* transcripts post exercise and the increased volume density of subsarcolemma mitochondria after thirty repetitions of

the exercise stimulus with training supports that transcript expression post exercise reflects exercise induced mitochondrial plasticity (Desplanches et al. 2014).

Another important factor, vascular endothelial growth factor (*VEGF*), causes increases in vascular permeability, endothelial cell proliferation, and angiogenesis. Breen et al. (1996) showed that mRNA levels of angiogenic growth factors such as *VEGF* in the gastrocnemius of rat were significantly increased after a bout of endurance exercise. The same was found after a period with chronic electrical stimulation during exercise (Hang et al. 1995) indicating that muscle activity leads to an increase in mRNA levels for angiogenic growth factors. The findings support the notion that transcript level alterations in skeletal muscle after a single bout of endurance exercise are a proxy for the subsequent adaptations with repetition of an endurance stimulus (Desplanches et al. 2014; Flück et al. 2006).

Muscle transcript expression after endurance exercise is graded with regard to metabolic stress and modulated by oxygenation (Schmutz et al. 2010). We hypothesized that the exercise-induced alteration in expression of genes of pro-angiogenic and oxygen-dependent factors are modulated by *ACE* activity due to its influence on muscle perfusion (Brothers et al. 2006). We assumed that pro-angiogenic factors such as *VEGF*, *Tenascin-C*, *Angpt1* and *Angpt1R* are increased post exercise while oxygen-dependent factors such as *COX4I*, *COX4I2* and *HIF-1α* are blunted after exercise in subjects taking an *ACE* inhibitor. This was tested in biopsies of knee extensor muscle within 3 hours after a single bout of bicycle exercise.

Methods

Experimental design

The study has been approved by the ethics board of the Manchester Metropolitan University. Written informed consent was obtained from every participant.

To test our hypothesis we carried out a pharmacological intervention with humans that were characterized anthropometrically. Fourteen Caucasian male subjects, non-specifically trained, took part in the study carrying out a maximal exercise test to characterize aerobic fitness and a single bout of one-legged exercise where we collected muscle and blood samples.

Table 1: Participants details. Charataristics of the participants in the *ACE* inhibition and control group. Values are mean ± SEM.

	ACE inhibition group		Control group	
Age (years)	30.3	± 2.72	23.3	± 1.33
Height (m)	1.76	± 0.04	1.80	± 0.02
Mass (kg)	75.4	± 4.6	72.33	± 2.87
Exercise (h/week)	10.4	± 1.83	3.2	± 0.63
VO_2-peak (l/min) (2-legged test)	3.72	± 0.74	3.65	± 0.32
VO_2-peak (l/min) (1-legged test)	3.28	± 0.21	3.15	± 0.62
Blood pressure at rest (mm HG)				
Systolic	130.8	± 7.1	118.9	± 1.75
Diastolic	71.4	± 4.1	71.4	+ 1.70
Systolic after *ACE* inhibition	129.8	± 4.1		
Diastolic after *ACE* inhibition	69.6	± 3.5		

Anthropometric characterization

In an entry test peak VO_2-peak was established with a maximal exercise test and subjects height and mass were measured. A mucosal mouth swab to establish the *ACE* genotype was taken by twirling/rubbing an ear bud against the inner cheek wall. Blood pressure was measured in a calm place in seated position with a sphygmomanometer after the subject has been sitting for at least 15 minutes. Seven of the participants were asked to consume daily an *ACE* inhibitor (ACE inhibition group: Lisinopril, 10 mg/day, Zestril, AstaZeneca) in the morning for three days including the day of the exercise test, the other group (control group) was not asked to use the *ACE* inhibitor. On the testing day subjects reported fasted to the laboratory and performed a single-leg standardized exercise test. Venous blood and a biopsy sample from *vastus lateralis* muscle were collected by a physician (SW). Blood collection was done via venipuncture from the upper arm before and immediately after the bout of exercise, while the subjects were in a sitting position. 5ml of blood was drawn from the Cephalic vein into a tube sprayed with dry EDTA (K2E BD Vacutainer, Belliver Industrial Estate, Phymouth, UK) placed on ice. A 2ml aliquot was rapidly processed for the measure of AngII.

Biopsies were collected under local anesthesia (subcutaneous injection of 1ml 2% Lidocaine) before and 3 hours post exercise from the belly portion of *vastus lateralis* muscle with a needle device (TSK Acecut 11G, UK biopsy).

Exercise tests

Maximal test

Two-leg exercise aerobic capacity was measured with ergospirometry during a cycling exercise test on an Excalibur bicycle (Lode Groningen, The Netherlands). Expired air was measured breath by breath (Oxycon alpha, Jager GmbH, Wurzburg, Germany) and heart rate using a heart rate belt (Accurex Plus, Polar Electro Finland, Kempele, Finland). The test started at 40 Watt and subjects were asked to cycle at 80 revolutions per minute (rpm). The power was increased every two minutes with 30 watts until the subject could no longer maintain a cadence higher than 60 rpm with verbal support.

One-legged exercise test

Subjects completed the one leg exercise test with their dominant leg and the shoe taped with duct tape to the pedal on an Excalibur bicycle (Lode Groningen, The Netherlands). The protocol of the exercise test was a 3 minutes warm-up at 50 Watt, followed by 25 minutes at 1.2-times body mass in Watts and ending with a ramp up of 10 Watt/min until exhaustion. See figure 1 for the characteristics of the stimulus. The subjects cycled at a cadence of 80 rpm and the test ended when in the last phase the cadence dropped below 60 rpm. This protocol was chosen to maximize the metabolic stimulus for recruited muscle groups, without central aspects posed a limitation that may interact with the systemic effects of *ACE* activity on blood pressure and perfusion.

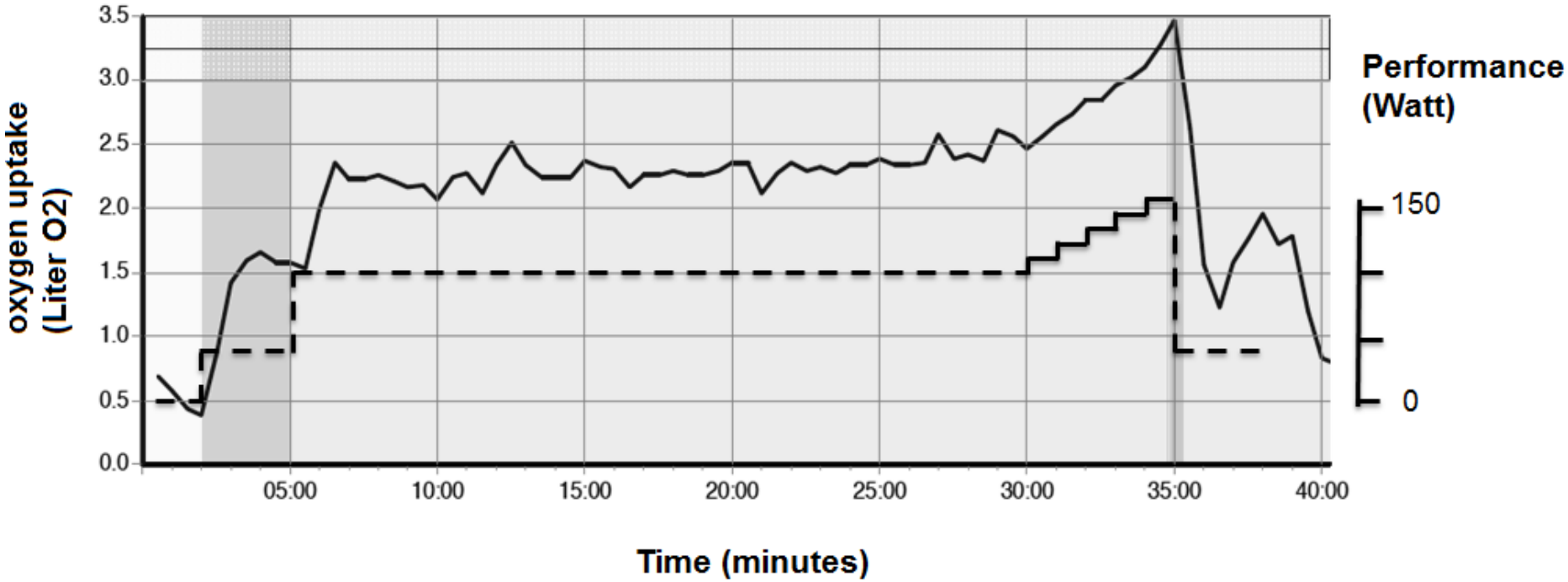

Figure 1: One-legged exercise. Line graph depicting the characteristics of the one-legged exercise stimulus. Oxygen uptake (continuous line) and the work rate during the warm-up, constant load, and ramp phases (stippled line) are shown. A clear peak of intensity can be identified.

ACE genotyping

DNA was extracted from mucosal samples using a custom designed protocol. The mucosal mouth swabs were extracted with 800 μl of methanol with vortexing and left to evaporate in air under a stream of nitrogen gas. The pellet was resuspended in 100ml of sterile water and stored at -20ºC.
ACE genotyping was carried out with Polymerase chain reaction (PCR) as described by Evans, (1994). The primers corresponded to those established previously for the identification of the ACE-I/D genotype (for details see Genbank number X62855): detection of the 83-bp-amplicon specific to the absence of the insertion sequence (i.e. the D-allele) was achieved by a combination of ACE1 (5'-catcctttctcccatttctc-3') and ACE3 (5'-atttcagagctggaataaaatt-3') primers. ACE2 (5'-tgggattacaggcgtgatacag-3') and ACE3 (5'-atttcagagctggaataaaatt-3') primers were applied to detect the 66-bp-amplicon specific for the I-allele in intron 16 of the *ACE* gene .
PCR reactions were run with a mix of the three primers using Sybr Green master mix (Applied Biosystems) on a Applied Biosystems Real Time PCR system (SepOnePlus, Life Technologies). This involved 45 standard cycles of denaturing at 95ºC for 15 sec followed by annealing and extension at 55ºC for 1 min. Amplicon identification followed using a melting curve analysis between a temperature range of 70ºC to 80ºC. The identity of the amplified sequences was validated by sequencing of the PCR products with the specific primers (Microsynth, Balgach, Switzerland). Subsequently, the presence of the short amplicon for the I-allele was identified based on a lower melting temperature (73.5ºC; 72.5 - 74ºC) compared to the longer D-allele (75.5ºC; 74.5 - 76.5ºC) respectively.

Assessment of AngII levels

Blood (2 ml) was withdrawn from the venous cannula into vacutainers containing 60µl AngII inhibitor cocktail, comprising 13.35µl of O-Phenanthroline and Pepstatine A in DMSO mixed with 46.65µl of EDTA and PHMB in aqueous solution (SPI bio, Bertin pharma, Versailles, France). The samples were immediately centrifuged at 10,000 rpm (3,000 g) and 4ºC for 12 min. The supernatant was separated, snap frozen in liquid nitrogen and stored at -80ºC until analysed. Plasma was extracted with C18 phenyl cartridge which were conditioned with 2ml of methanol and then rinsed with 2ml of water. Cold plasma (0.9ml) was rapidly passed through the cartridge and subsequently washed with 1ml of water. Absorbed angiotensins were eluted with 1ml of methanol into conical polypropylene tubes. The eluate was evaporated to dryness by means of a nitrogen gas stream at room temperature and the residue was stored at -20^{o}C. AngII was assessed with the AngII enzyme immunoassay kit (SpiBio, Montigny Le Bretonneux, France). Briefly, the plasma samples were incubated for 1 hour with 100µl of EIA buffer (reconstituted the EIA buffer vial with 50ml of distilled water), for 5 minutes with 50µl of glutaraldehyde (100 ·l of gluteraldehyde diluted in 0.125ml of concentrated Wash buffer and 4.878ml of distilled water), for 5 minutes with 100µl of borane-trimethylamine (borane-trimethylamine vial diluted in 2.5ml of 2N HCL and 2.5 ml of methanol) on a rocker platform, and with 100µl of anti-AngII IgG tracer (reconstituted the anti-antiotensin II-IgG tracer in10ml of EIA buffer) at 4^{o} overnight. On the next day the samples were incubated with 200µl of Ellman's reagent (Ellman's reagent vial in 1ml of concentrated Wash buffer and 29ml of distilled water). The plate was run with a

single quick read on an Absorbance Microplate Reader (ELx800, BIO-TEK) with wavelength 405 after 30 minutes, 1 hour and 2 hours of incubation.

After plotting the absorbance of each standard point versus the concentration, the AngII concentrations were calculated by interpolating from this standard curve. In order to reduce variability, AngII values were normalized by dividing every value by the median of the pre values.

Transcript measures

Total RNA was isolated from $10mm^3$ of biopsy samples in 20um sections using an RNeasy mini kit (Qiagen, cat N^o 74101) and Proteinase K (Qiagen, cat N^o 19131) as described by Schmutz et al. (2010). Concentration and quality of RNA were determined using a NanoDrop USV-99 AGTGene (Labgene Scienfitic SA, Switzerland).

cDNA was reverse transcribed from 600 ng of RNA using a OMNIscpript Kit (Qiagen, cat N^o 205110) with random hexameres according to the manufactures' instructions. RT-qPCR was carried out for 8 candidate genes (*Tenascin-C*, *COX4I1*, *COX4I2*, *HIF-1a*, *Angpt1*, *Angpt1R, VEGF*, and *GAPDH*) and the reference gene for normalization (28S r RNA). cDNA corresponding to an estimated input of 0.6ug (0.06ug for 28s) was dispensed into each well with a final reaction volume of 10ul. Pooled primer pairs (200nM each) and KAPASSybr FAST Master MIX 2X (Kapa Biosistems, KK4600) were performed on Eco^{TM} Thermal and Optical system (Illumina, Labgene Scientific SA, Switzerland). Primer sequences for each gene are listed in Table 2. Each sample was amplified in duplicate using the following conditions: preheating at 95ºC for 2 minutes, followed by 45 cycles at 95ºC for 15 seconds ant 60ºC for 1 minute. The

calculation of transcript amounts were analyzed with the ΔCT method but taking the individual efficiency into account. The resulting values were standardized to those of 28S.

Table 2. Summary of the reference genes and Primers sequence.

Primer	Sequences
28S rRNA	Forward Primer: 5'ATA TCC GCA GCA GGT CTC CAA-3' Reverse primer: 3'-GAG CCA ATC CTT ATC CCG AAG-5'
COX-1	Forward Primer: 5'-CTA TAC CTA TTA TTC GGC GCA TGA -3' Reverse Primer: 3'-CAG CTC GGC TCG AAT AAG GA-5'
COX-4I1	Forward Primer: 5'-GCC ATG TTC TTC TTC ATC GGT TTC-3' Reverse Primer: 3'-GGC CGT ACA CAT AGT GCT TCT G-5'
HIF-1 α	Forward Primer: 5'-TAG TGA ACA GAA TGG AAT GGA GCA A-3' Reverse Primer: 3'-TTT TTG GAC ACT GGT GGC TC5'
Angpt1	Forward Primer: 5'-CGC TGC CAT TCT GAC TCA CAT A-3' Reverse Primer: 3'-CGG TTA TAT CTT CTC CCA CTG TTT TC-5'
Angpt1R	Forward Primer: 5'- GTT GGC CTT TCT GAT CAT ATT GC-3' Reverse Primer: 3'-GGT TCT TCC CTC ACG TTT TGG-5'
Tenascin-C	Forward Primer: 5'-TAC CAA AAA GCA GTC TGA GCC ACT GGA AAT AAC CCT ACT TGC CCC CGA ACG TAC CAG GGA CAT AAC AGG TCT CAG AGA GGC TAC TGA ATA C-3' Reverse Primer: 3'-CAT AAG TCA TCG GAG AGA CTC TGG ACA ATT CAG GGA CCA TGC AAG CCC CCG TTC ATC CCA ATA AAG GTC ACC GAG TCT GAC GAA AAA CCA T-5'
VEGF	Forward Primer: CAT GGC AGA AGG AGGAGG GCA GAA TCA Reverse Primer: ATC TTC AAG CCA TCC TGT GTG CCC CTG
GAPDH	Forward Primer: 5'-GGA GCG AGA CCC CAC TAA CA-3' Reverse Primer: 5'-GCC TTC TCC ATG GTG GTG AA-5'

Bp, base par; PCR, Polymerase Chain Reaction; HIF-1a, Hypoxia-Inducible Factor-1a; COX, cytochrome C oxidase; Angpt, angiopoetin; VEGF, Vascular Enothelia Growth Factor; GPDH, glyceraldehydes-3-phosphate dehydrogenase

Statistics

Data were organised in MS-Excel and exported into SPSS 19.0 for statistical testing. Differences in blood pressure and AngII were analyzed with a paired samples t-test. Pre/post changes and fold changes in transcript expression of *COX4I1, COX4I2*, *HIF-1α*, *Angpt1* and *Angpt1R* between the *ACE* inhibition group and control group were assessed with a multiple analysis of variance and a two-factor ANOVA respectively.Significance of a difference was declared at $p<0.05$.

Results

The *ACE* genotypes of the subjects: *ACE* inhibition group (ACE-DD: 5, ACE-ID/II: 7); control group (ACE-DD: 5, ACE-ID/II: 1). Anthropometry (mass, height, age), VO_2max and resting blood pressure of the subjects of the two groups did not differ significantly (Table 1). There was no difference in systolic ($p=0.16$) or diastolic blood pressure ($p=0.94$) between the study groups. Intake of *ACE* inhibitor did not affect systolic ($p=0.90$) or diastolic ($p=0.73$) blood pressure at rest (Table 1).

There was a trend for an effect for the intake of *ACE* inhibitor on the fold changes in transcript expression post exercise ($p=0.09$, figure 2). Fold changes in transcript expression in the vastii was significantly higher 3 hours post exercise in the control group for *COX4I2* ($p=0.03$) and *HIF-1α* ($p=0.05$). The level of *COX4I2* was increased 3 hours post exercise in the control group ($p=0.04$) but not in the *ACE*

inhibition group (p=0.91). Fold changes in transcript expression post exercise was significantly larger in the absence of *ACE* inhibitor intake of the pro-angiogenic transcript *VEGF* (p=0.04). A trend for such an effect was seen for *Tenascin-C* (p=0.09).

AngII levels were 1.6 times and glucose 1.20 times significantly increased after exercise under oral intake of *ACE* without significant increases in the control group (see table 3).

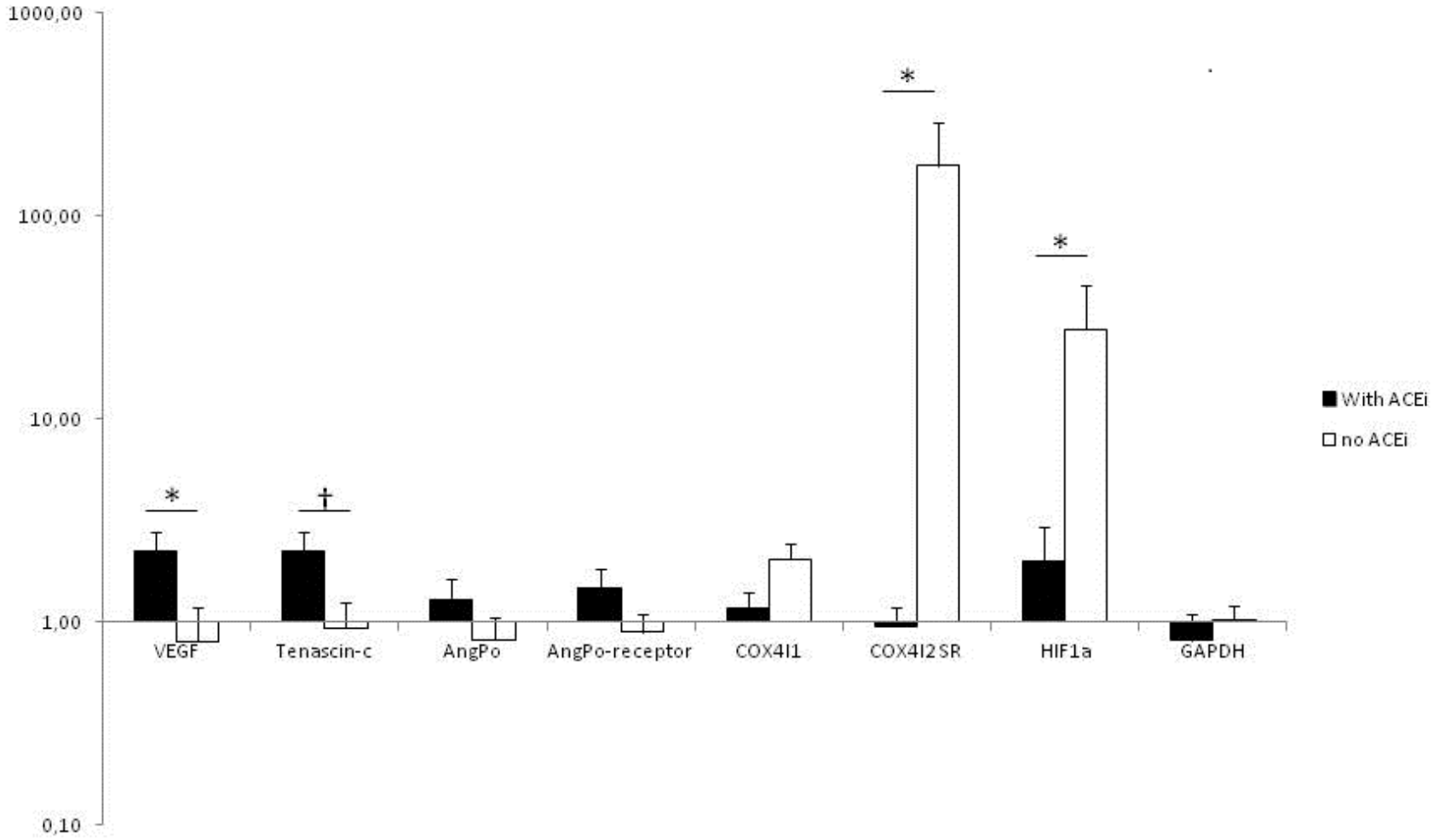

Figure 2: *Transcript expression in the vastii 3 hours post exercise with or without oral intake of ACE inhibitor. AngPo*: Angiopoietin 1; *COX4I1*: Isoform 1 for subunit 4 of cytochrome c oxidase; *COX4I2*: Isoform 2 for subunit 4 of cytochrome c oxidase; *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase; *HIF-1*α: hypoxia-inducible factor α-subunit; *VEGF*: Vascular endothelial growth factor. * and † denote significant difference ($p<0.05$) andtrend ($0.05 \leq p<0.10$; Repeated measures ANOVA).

Table 3: AngII and glucose levels pre and post exercise and the fold increases.

	Group	Pre		Post		Fold	
AngII values (pg/mol)	ACE inhibition	17.4	±3.7	25.0	±3.9	1.6†	±0.4
	Control	15.2	±3.7	18.2	±2.2	1.7	±0.4
Glucose (mmol/l)	ACE inhibition	2.33	±0.13	2.85*	±0.19	1.20†	±0.53
	Control	4.70	±0.48	4.63	±0.76	1.02	±0.20

Values are mean ± SEM paired samples t-test. * denotes significant different from pre *ACE* inhibition ($p<0.05$), † denotes significant increase post exercise ($p<0.05$).

Discussion

Three hours after exhaustive cycling exercise the pro-angiogenic factor *VEGF* and *Tenascin-C* were increased in the *ACE* inhibition group, but not in the control group.

Also in the study of Gavin et al. 2000 they found *VEGF* mRNA expression both in the group who took the first generation *ACE* inhibitor captopril as in the control group after an hour of moderate intense exercise (≈50% $VO_{2\text{-max}}$). In that study the post exercise *VEGF* levels were not significantly different from the group that took the placebo.

Flueck (2009) reviewed that endurance training under hypoxia enhances the structural components of local aerobic capacity by increasing mitochondrial protein expression. The low intramuscular oxygen levels in the muscle in combination with contraction-related signals are the driving force for angiogenic and mitochondrial protein expression with endurance training. Involvement of signaling to muscle remodeling with low oxygen content with exercise is illustrated by the modulation of the master regulator of hypoxia-regulated gene expression, the dimeric transcription factor HIF-1 (Semenza et al. 2004). Hypoxia-dependent regulation by HIF-1 is turned off in well- oxygenated conditions by the degradation of its alpha-subunit, *HIF-1*α. When the hypoxia drops to a critical level, *HIF-1*α is stabilized (Flueck et al. 2009). In our study *HIF-1*α transcript levels were increased in the control group, while they were blunted in the *ACE* inhibition group. The same was observed for *COX4I2*. We have reported before that *HIF-1*α and *COX4I2* transcript levels are regulated in a hypoxia-modulated manner after two-legged endurance type bicycle exercise (Desplanches et al. 2014). Thereby *COX4I2* transcript levels were increased

24 hours after a single exercise bout at 65% of respective peak aerobic power output in ambient hypoxia equivalent to 4,000 m above sea level. Exercise in hypoxia produces a pronounced drop in muscle oxygenation (reviewed in Desplanches et al. 2014). This emphasizes that enhanced *COX4I2* mRNA expression in exercised muscle constitutes a marker of local hypoxia (Fukuda et al. 2007). These considerations support the notion that the modulation of muscle's transcript regulation by lisinopril is related to muscle oxygenation during exercise.

The AngII values were 1.6 times higher after exercise in the *ACE* inhibition group and not significantly increased in the control group. This increase in AngII is comparable with the study of Fagard et al. (1985). The increase in the *ACE* inhibition group as well as the higher increase in this group compared with the control group could indicate an incomplete inhibition of *ACE* during and after exercise. The difference in AngII levels between the two groups can be explained by an ACEinhibition at rest and so low AngII levels and an incomplete inhibition during exercise. This is supported by the study of Aldigier et al. (1993) where they found a higher increase of AngII in the *ACE* inhibition group during exercise compared to the control group.

The data in this study point at an effect of commonly used anti-hypertensive treatment on the muscle response to exhaustive bicycle type endurance exercise and supports the view that an angiotensin-regulated mechanism dictates the hypoxia-specific gene response in peripheral muscle to endurance exercise. Our finding that lisinopril increases pro-angiogenic factors associated with the capillaries in the interstitium, i.e. *VEGF* and *Tenascin-C* (Milkiewicz et al. 2001, Flück et al. 2000) and

inhibits the increase in oxygen dependent transcripts which are thought to mainly situate in muscle fibres (*COX4I2*, *HIF-1α*) indicates that there is a shift in the activation of the gene program from muscle fibres to the surrounding interstitium under oral intake of *ACE* inhibitor. As *ACE* inhibitors in combination with endurance type of exercise is widely prescribed to people with hypertension, our findings can have ramifications for improving the current anti-hypertensive treatment.

A limitation of this study is that it is not built on a cross-over design. The absence calls for a further study to confirm our findings and expose the functional implication of our observation. As this study is based upon a single exercise bout, the protein levels corresponding to the measured transcripts (for instance *VEGF*) were not assessed, because protein levels are expected to change as a response to endurance training rather than a single exercise bout.

Conclusion and perspective

Exercise-induced alterations in gene transcripts are partly under the control, or modulated, by ACE-dependent pathways. This exercise induced mechanism has rarely been valued in pharmacological studies of hypertension and exercise rehabilitation (Böhm et al. 2008). Awareness of the existence of such a mechanism could develop a major impact on current Health Care practice, particularly in the situation of exercise rehabilitation when one wants to exploit the sympatholytic and angiogenic benefits of physical therapy. This relationship can have major economic and clinical repercussions as inhibiting *ACE* therapies are the current default for hypertensive patients.

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Author contributions

Author contributions: Sv.G. and M.F. conception and design of research; Sv.G. (detailed below), S.R., S.W., and M.F. performed experiments; Sv.G. and M.F. analyzed data; Sv.G. and M.F. interpreted results of experiments; Ad.H. and M.F. funding; Sv.G and M.F. prepared figures; Sv.G. and M.F. drafted the manuscript; Sv.G., H.D., Ad.H., and M.F. edited and revised the manuscript.

Experiments performed by Sv.G.: Taking informed consent, height, weight, blood pressure, glucose, cycling ergometer tests, blood processing, *ACE* genotyping, AngII assessement, transcript measures

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Chapter 4

Association of *Tenascin-C* with exercise-induced angiogenesis and angiotensin-converting enzyme (ACE) activity

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ABSTRACT

Endurance training can increase muscle capillary density in human skeletal muscle. We hypothesized that this process depends on capillary adhesion modulation through elevated expression of *Tenascin-C* with exercise-induced vasodilatation. To investigate this possibility, we examined whether (I) training-induced capillary remodeling in vastus lateralis muscle of healthy, Caucasian, male subjects is affected by the single-nucleotide polymorphism (SNP) rs2104772 that is predicted to affect *Tenascin-C*'s adhesive properties due to a thymidine (T)-to-adenosine (A) exchange at position 44,513 in the *Tenascin-C* gene. We further analyzed whether (II) *Tenascin-C* expression in exercised muscle depends on *ACE* activity and the training state, which both increase muscle perfusion capacity. *Tenascin-C* transcript levels in vastus lateralis were elevated 8 hours after exhaustive one-legged bicycle exercise in trained (n = 7) but not in untrained subjects (n = 7). Intake of *ACE* inhibitor Lisinopril (n = 7) increased *Tenascin-C* and *VEGF*-A transcript levels in untrained subjects after the bicycle exercise but failed to reduce blood pressure and exercise-induced serum angiotensin 2 concentration while increasing *ACE* activity in muscle. Six weeks of bicycle endurance training (n = 36) elevated the concentrations of the small (+128%) and large (+77%) *Tenascin-C* isoform. Likewise, capillary-to-fiber-ratio (+13%), capillary density (+10%), and mitochondrial volume density (+24%) were increased after training in an rs2104772 dependent manner (P < 0.01). Capillary-to-fiber ratio was reduced in T/T genotypes (14%). Our findings support a role of *Tenascin-C* expression in the stabilization of capillaries, which depends on enhanced angiotensin 2 concentration under concomitant inhibition of vasoconstriction during endurance work.

INTRODUCTION

Endurance training—i.e., the repeated contraction of skeletal muscle at a moderate to high load until fatigue—increases mitochondria and capillary volume density, thus improving the myocellular energy supply (Mortensen et al. 2012, Wibom et al. 1992, Zumstein et al. 1983). The first weeks of training typically induce 20–40% increases in capillary and mitochondrial volume density (Hoppeler et al. 1985). The underlying mechanisms involve metabolic and mechanical factors, whereby stimuli that influence extracellular matrix architecture and composition seem to pronouncedly impact on capillary density and capillary-to-fiber ratio (Badr et al. 2003, Prior et al. 2004). Angiotensin-mediated vasoconstriction limits blood flow in resting muscle and must be overcome with the onset of exercise to allow perfusion of contracting muscle tissue (reviewed in (Brothers et al. 2006, Clifford et al. 2004, Korthuis 2011, Wunsch et al. 2000). Endurance training exerts a positive influence on exercise-induced muscle perfusion (reviewed in (Husain et al. 2014, Mortensen et al. 2014), by improving endothelium-dependent vasodilatation of arterioles and capillaries (Korthuis 2011). Collaterally with intense exercise, however, an increase in serum angiotensin 2 concentration can be observed, potentially reducing blood flow to metabolically-inactive tissues (Brothers et al. 2006, Korthuis 2011, Staessen et al. 1987) and exerting direct actions on endothelial cells in the perfused capillaries (Clapp et al. 2009). The inhibition of angiotensin's action on arterioles therefore may be a main contributor to increasing shear stress to capillary walls, which is believed to drive angiogenesis with exercise (Hudlicka et al. 1992) and may conspire with the pro-angiogenic action of angiotensin 2 (Clapp et al. 2009). This relationship is highlighted by the enhanced muscle capillary density in subjects with increased

expression of angiotensin-converting enzyme (ACE), which produces the vasoconstrictor angiotensin 2 (Vaughan et al. 2013).

Studies in rodents show that the anti-adhesive extracellular matrix protein *Tenascin-C* is a mechanoregulated governor of angiogenic gene expression in cardiac and skeletal muscle (Ballard et al. 2006, Fluck et al. 2008). Reflecting this role, *Tenascin-C* is expressed in migrating endothelial cells and branching sites of blood vessels (Hahn et al. 1995, Jarvinen et al. 2003, Mackie et al. 1992, Mustafa et al. 2012). *Tenascin-C* expression is induced by the vasoconstrictor peptide angiotensin II in cultured smooth muscle cells, and is increased in vascular structures in hypertension (Mackie et al. 1992, Sharifi et al. 1992), suggesting that *Tenascin-C* mediates the effect of angiotensin II on pathological blood vessel growth. However, this hypothesis has never been tested in vivo, particularly as it relates to physiological angiogenesis after endurance exercise. Nor has any study characterized whether endurance exercise affects other metabolic processes related to capillary growth, such as mitochondrial biogenesis, via a *Tenascin-C* related mechanism.

Here we used a genetic approach with the aim of identifying the role of *Tenascin-C* in endurance training-induced remodeling of capillary supply lines in the knee extensor muscle, as well as the interactions of *Tenascin-C* with the angiotensin converting enzyme. We focused on the single-nucleotide polymorphism rs2104772 (A/T) in exon 17 of the *Tenascin-C* gene, which impacts the elastic/adhesive properties of *Tenascin-C* in subjects with the A/A genotype (Matsuda et al. 2005, Saunders et al. 2013). We investigated I) whether subjects with the A/A genotype of rs2104772 demonstrated a blunted muscle capillary density and capillary-to-fiber

ratio response following training, II) whether the identified effects were reflected by an altered *Tenascin-C* expression response to a single bout of endurance exercise and, finally, III) whether this response and expression of *VEGF*-A that is associated with contraction-induced angiogenesis through an angiotensin-dependent mechanism (Amaral et al. 2001) was influenced by *ACE* activity.

METHODS

Design

This study included two groups of white Caucasian subjects. Group 1 performed a single bout of bicycle exercise with or without oral intake of the *ACE* inhibitor Lisinopril at Manchester Metropolitan University in the United Kingdom. Group 2 performed endurance training on a stationary bicycle ergometer at the University of Berne in Switzerland.

Group 1 included 21 healthy, young male subjects of white British descent, 7 of which were endurance-trained. One week prior to the single bout of exercise, tests were conducted to estimate single- and two-legged aerobic performance. Three days before the bout of exercise subjects were asked to reduce physical activity. 7 randomly chosen untrained subjects were asked to consume the *ACE* inhibitor Lisinopril (10 mg daily; Zestril, AstraZeneca) in the morning, including on the day of the exercise test. Subjects reported to the laboratory in the fasted state. Blood pressure was measured in a calm place in seated position with a sphygmomanometer after the subject has been sitting for at least 15 minutes.

Subjects then performed a standardized one-leg exercise test to voluntary exhaustion. This intervention was chosen in order to maximize the metabolic stimulus for recruited muscle groups, without that central aspects pose a limitation that may interact with the systemic effects of *ACE* activity on blood pressure and perfusion (McPhee et al. 2009). Immediately before, 3 hours after exercise, biopsy samples were collected from the vastus lateralis (knee extensor) muscle from both the resting and exercised legs. In untrained subject not consuming Lisinopril a further 8 hours biopsy was collected. Biopsies were used for the characterization of *Tenascin-C* transcript expression using RT-PCR.

Group 2 included 36 healthy, non-specifically trained, young, male subjects of Swiss descent. The subjects were asked to refrain from extra physical activity for 2–4 weeks before the start of the study and endurance training program. Basic anthropometry and aerobic performance were assessed by a single bout of bicycle ergometer exercise two days before and after training, and muscle biopsies were collected from the vastus lateralis muscle. The biopsies were used for characterization of tissue composition using morphometric and molecular analysis. Molecular analysis included characterization of *Tenascin-C* protein expression and genotyping of the SNP rs2104772 within the *Tenascin-C* gene.

Ethics

The study protocols were approved by the ethics board of Manchester Metropolitan University (group 1; Manchester, United Kingdom) and the ethics committee of the Canton of Berne (group 2; Berne, Switzerland). All investigations were performed in accordance with the ethical standards of the 1964 Declaration of Helsinki. Written informed consent was obtained from every participant. Previous publications report the anthropometry and exercise performance and the consequences of a single bout of exercise and of training on volume density of mitochondria and capillarity for group 2 (Luthi et al. 1986, Schmutz et al. 2010, Schmutz et al. 2006, Suter et al. 1995).

Endurance test

In both groups, aerobic performance was assessed with ergospirometry. Two weeks before the first bout of exercise, subjects were familiarized with the test equipment. Exercise tests were conducted using a bicycle ergometer (Ergoline 800S; Ergoline GmbH, Bitz, Germany). Expired air was analyzed with breath-by-breath measurements (Oxycon alpha; Jäger GmbH, Würzburg, Germany) and heart rate was monitored using an Accurex Plus chest belt (Polar Electro Finland, Kempele, Finland). Starting at 40 watts, the workload was increased by 30 watts every 2 min until the subjects could no longer maintain a cadence of over 60 rpm. Group 1 underwent an additional test to determine aerobic performance of one-legged exercise.

Endurance training

Subjects trained for 6 weeks, with five 30-minute sessions per week, maintaining 65% of their maximal aerobic performance. Training intensity was monitored and adjusted based on heart rate. The training workload was increased as necessary to maintain a constant training heart rate corresponding to 83 ± 1% of the individual's maximal heart rate in the first training week, and to 90 ± 2% in the sixth training week. Before and after training, tissue was collected from the belly portion of the vastus lateralis muscle using a modified Bergstroem biopsy device with local anesthesia administered via subcutaneous injection of 1 mL 2% lidocaine, as previously described (Hoppeler et al. 1985).

One-legged exercise

Subjects completed a bout of one-legged exercise (using their dominant leg) on the stationary bicycle at a set cadence of 80 rpm under ergospirometry (Ergometrics Ergoline 800; Jaeger, Bitz, Germany). Saddle length was set to the same value used for two-legged exercise. The shoe of the dominant leg was attached to the pedal with duct tape, while the other leg rested on the frame in the middle of the ergometer or on a chair next to the bicycle. Subjects performed a warm-up at 15% of the calculated two-legged Pmax. Next, the subjects exercised for 25 minutes at 30% of the two-legged Pmax, after which the set intensity was ramped up in 10-Watt increments each minute until exhaustion. At the end of the exercise period, a 3-minute cool-down phase was allowed at 15% of the calculated two-legged Pmax. $\dot{V}O_2$, CO_2, and ventilation were monitored with a MetaLyser® 3B

system operated with MetaSoft® software (Cortex Biophysik GmbH, Leipzig, Germany), and peak $\dot{V}O_2$ and maximal RER were determined.

Biopsies were collected from the belly portion of the vastus lateralis muscle using a TSK Acecut 11G needle device (UK biopsy) under local anesthesia via subcutaneous injection of 1 mL 2% lidocaine. Before and immediately after the bout of exercise, 5 mL of blood was drawn from the cephalic vein into a K2E BD Vacutainer® tube sprayed with dry EDTA (Belliver Industrial Estate, Phymouth, UK). The tube was immediately placed on ice, and a 2-mL aliquot was rapidly processed to measure the angiotensin 2 concentration.

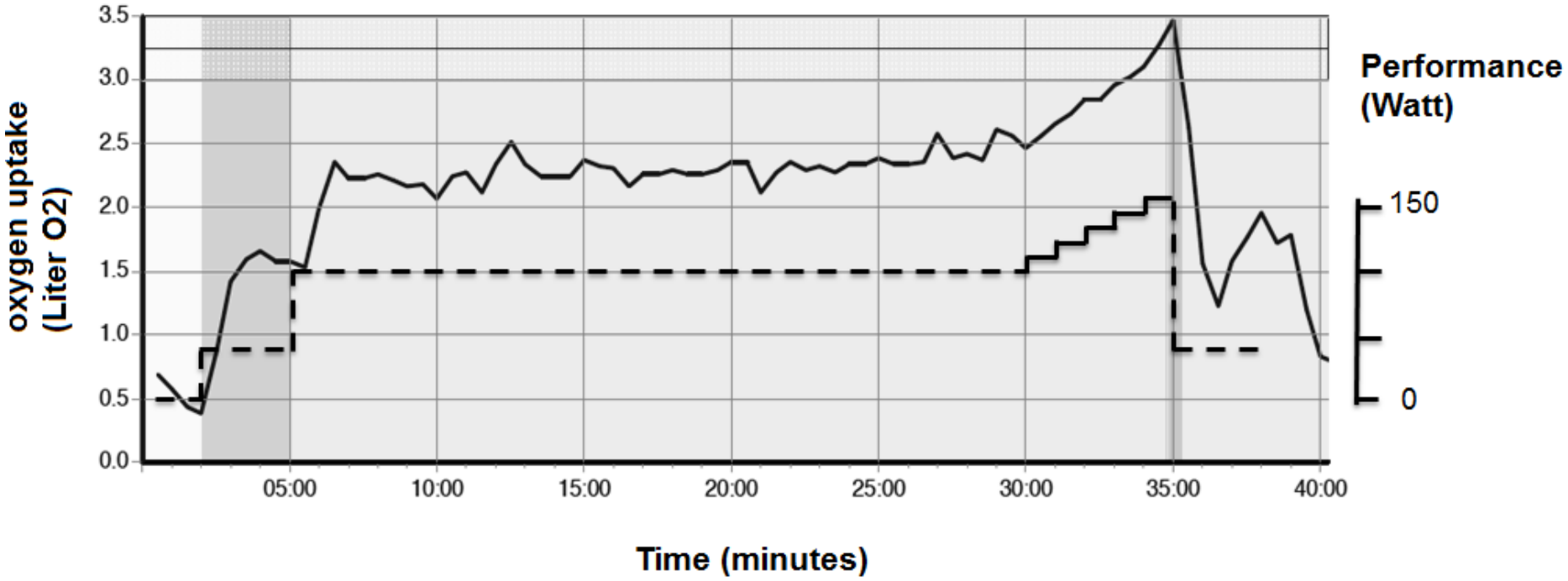


Figure 1. One-legged exercise protocol. Line graph depicting the characteristics of the one-legged exercise stimulus. Oxygen uptake (continuous line) and the work rate during the warm-up, constant load, and ramp phases (stippled line) are shown. A clear peak of intensity can be identified

Quantification of serum angiotensin 2 concentration

A 2-mL aliquot of venous blood was pipetted into a 2-mL Eppendorf tube on ice, which contained an inhibitor cocktail to prevent angiotensin 2 degradation (0.44 mM o-phenanthroline, 25 mM EDTA, 1 mM p-chloro-mercuribenzoic acid, and 0.12 mM pepstatin A). The samples were centrifuged for 15 minutes at 3000 rpm at 4°C, and then the supernatant was transferred to a new 2-mL tube and stored at −80°C until further processing. Subsequently, the pre- and post-samples were thawed and enriched for the hydrophobic angiotensin 2 peptides using SPE reverse-phase C18 cartridges (Cayman Chemical Company, Ann Arbor, MI, USA). The bound peptides were extracted with methanol into a glass tube, and dried down using a steam of nitrogen. The residue was dissolved in 250 µL EIA buffer, and two 100-µL aliquots were analyzed using the Ang II Enzyme Immunoassay kit (SPIBio Bertin Pharma, Montigny le Bretonneux, France) following the manufacturer's instructions. After 10, 30, and 60 minutes, the intensity of color development was recorded by spectrophotometry using an Automated Microplate Reader ELx800 (BIO-TEK® Instruments, Winooski, VT, USA). For each sample, the angiotensin 2 concentration was calculated using an angiotensin 2 standard curve. To establish the sensitivity and reproducibility of the assay, we initially ran test experiments with spiked angiotensin 2.

Analysis of muscle tissue

Muscle biopsies of group 2 were split in two portions. One portion was processed for electron microscopy to assess micro-anatomical characteristics of the

muscle cells and organelles based on morphometric analysis as previously described (Suter et al. 1995). The other portion was analyzed for *Tenascin-C* protein, SNP rs2104772 and *ACE* activity. Biopsies collected from group 1 were analyzed for muscle capillary density and capillary-to-fiber ratio and fiber cross-sectional area based on light microscopic measure of selected gene transcripts as previously described (Schmutz et al. 2010).

Genotyping

The targeted SNP rs2104772 was analyzed using high-resolution melt PCR (HRM-PCR). Genomic DNA was extracted from ~1-mm^3 cryosections of the muscle biopsy using the DNeasy Blood and Tissue Kit (Qiagen Cat. No 69504) following the manufacturer's protocol, essentially as previously described (45). DNA concentration and purity were measured using a NanoDrop USV-99 AGTGene (Labgene Scientific SA). Absorbance measurements at 260 and 280 nm indicated that the final DNA concentration ranged from 10 to 50 ng/μL and that the DNA was of high purity. DNA samples were diluted to a final concentration of 5 ng/μL and stored at −20°C until analysis. Genotyping was performed with HRM-PCR combined with sequencing. Available online tools (i.e., Primers-BLAST: http://www.ncbi.nlm.nih.gov/tools/primer-blast/; and Primer 3 output: http://primer3.ut.ee) were used to design oligonucleotide primers to target the *Tenascin-C* polymorphism (rsNCBI: rs2104772; SNP-44513-AT). The designed primers (5'-GATGAAGGGGTCTTCGACAA-3' and 5'-TCGCCTTCCTTTGCTTATTC-3') amplified a 168-bp fragment containing the SNP rs2104772.

The PCR reaction specificity was validated in experiments with negative and positive controls and by confirming the predicted PCR product size via agarose gel electrophoresis. This was followed by DNA band isolation and subsequent DNA sequencing by Microsynth (Balgach, Switzerland). The negative control was a non-template control reaction (NTC) with DNAse-free water and positive controls comprised genotyped DNA samples, including samples for each genotypic variant of rs2104772 (i.e., A/T, A/A, and T/T). The positive controls also served as internal references to generate a melting profile for screening unknown samples.

HRM-PCR reactions were run in duplicate. The reaction mix included 10 ng DNA template, 1× KAPA HRM FAST Master Mix (KAPA BIOSISTEMS, Labgene scientific SA, Châtel-St-Denis, Switzerland), 2.5 mmol $MgCl_2$, 0.2 µmol of each primer, and water up to a final volume of 10 µL. Amplification and melt curve analysis was then performed using a EcoTM Thermal and Optical system (Illumina; Labgene Scientific SA, Châtel-St-Denis, Switzerland). The reaction conditions were as follows: 3 minutes enzyme activation at 95°C; followed by 35 amplification cycles of 5 seconds denaturation at 95°C and 30 seconds annealing/extension at 60°C; and a final melting cycle of heating to 95°C, cooling to 55°C, and ending at 95°C. For each cycle, the fluorescent signal from the EvaGreen-dye contained in the 1× KAPA HRM FAST Master Mix was analyzed using EcoStudy software (Illumina, Labgene Scientific SA). This software automatically calculated and clustered the samples into three groups based on the melting profile, and the results were displayed as derivative normalized melting curves and peaks.

Transcript expression

Total RNA was isolated from a 10-mm^3 biopsy divided into 20-µm sections using an RNeasy Mini Kit (Qiagen, cat N° 74104) and Proteinase K (Qiagen, cat N° 19131) as described by Schmutz-Hoppeler (Schmutz et al. 2010). The isolated RNA was stored overnight at −20°C. The RNA concentration and quality were determined using a NanoDrop USV-99 AGTGene (Labgene Scientific SA, Switzerland).

From 600 ng RNA, cDNA was reverse transcribed using an OMNIscript Kit (Qiagen, cat N° 205110) that uses random hexamers, following the manufacturer's protocol. RT-qPCR was performed for *Tenascin-C*, *VEGF*-A and the 28S rRNA gene for normalization. Into each well was added cDNA corresponding to an estimated input of 0.6 µg (0.06 µg for 28S), with a final reaction volume of 10 µL. Pooled primer pairs (200 nM each) and KAPASybr FAST Master MIX 2X (KAPA BIOSISTEMS, KK4600) were added and the reaction was performed using an Eco™ Thermal and Optical System (Illumina, Labgene Scientific SA, Switzerland). The following primers were obtained from Microsynth (Balgach, Switzerland): *Tenascin-C* (5'-ACCTCTCTGGAATTGCTCCCA-3', 5'-CATCTGAAACTAGAAGGTTGTC-3'); *VEGF*-A (5'-CAT GGC AGA AGG AGGAGG GCA GAA TCA-3', 5'-ATC TTC AAG CCA TCC TGT GTG CCC CTG-3'); 28S rRNA (5'-ATA TCC GCA GCA GGT CTC CAA-3', 5'-GAG CCA ATC CTT ATC CCG AAG-3'). Each sample was amplified in duplicate using the following conditions: preheating at 95°C for 2 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Transcript amounts were determined with the ΔCT method, taking individual efficiency into account. The calculated values were standardized to the amounts of 28S.

Detection of Tenascin-C protein

From the biopsy material, total protein homogenate was prepared and subjected to SDS-PAGE and immunoblotting for *Tenascin-C* essentially as previously described (Fluck et al. 2008). Briefly, 10 mm3 of muscle tissue was cross-sectioned to 25 µm using a cryostat and mixed with ice-cold RIPA buffer that included 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2% Triton X100, 2 mM EDTA, 2 mM EGTA, one PhosStop tablet, and one tablet of Complete-mini EDTA-Free reagent (Roche Diagnostics GmbH, Mannheim, Germany). This mixture was homogenized using a Polytron®PT 1200E hand-held homogenizer (Kinematica AG, Lucerne, Switzerland). The amount of protein in the total homogenate was determined using the BCA method (Pierce, Rockford IL, USA) against bovine serum albumin (BSA) as a standard. The total homogenate was adjusted to a concentration of 2 µg per µL using Laemmli buffer (Biorad Laboratories AG, Cressier, Switzerland) and 2% mercaptoethanol, and the mixture was heated at 95°C for 5 min.

Next, 20 µg total protein was separated on hand-made 7.5% polyacrylamide gels in a Mini-Protean III electrophoretic system (BioRad Laboratories AG, Cressier, Switzerland). Samples of pre- and post-training pairs were loaded separately in adjacent lanes, with four sample pairs loaded per gel. Proteins were subsequently transferred onto nitrocellulose membrane (Protean, Amersham), the blotting efficiency was visualized by Ponceau S staining. Immunodetection was performed using the *Tenascin-C* antibody B28.13 (a gift from Prof. Matthias Chiquet) at a 1:50 dilution, and secondary horseradish peroxidase-conjugated anti-rabbit IgG raised in

goat (MP Biomedicals, #55676). The bands corresponding to the tagged proteins were detected using chemoluminescence (Femto kit; Pierce, Fisher Scientific AG, Wohlen, Switzerland) and recorded using a Chemidoc system with Quantity One software (Bio-Rad, Life Science Research, Hercules, CA, USA). Band signal intensity was estimated using the "volume rectangular tool" and was corrected versus the background of a band of equal height and size (area) from an empty sample lane. Background-corrected data were normalized to actin, and then normalized to the mean values of the pre-training sample from the same gel. Therefore, the final values reflect the relative expression levels per total muscle protein.

Measurement of ACE activity

The analysis of *ACE* activity in muscle tissue before exercise was carried out using a fluorometric assay as modified from the published method of Sentandreu and Todra (2006) (Sentandreu et al. 2006). In brief, 10 mm3 of biopsy material from *vastus lateralis* muscle was cross-sectioned in a cryostat and homogenate was prepared in ice-cold 0.1M KH2PO4 buffer using a Polytron®PT 1200E hand-held homogenizer (Kinematica AG, Lucerne, Switzerland). Protein concentration was determined using the BCA method (Pierce, Rockford IL, USA) against BSA as a standard.

Total homogenate corresponding to 35 ·g protein in 100 µl 0.1M KH2PO4 buffer was assessed by adding 200 µl of solution containing 0.45 mM of substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO2)-Pro; Bachem cat. no. M-1100) and 1.125M NaCl in 150mM Tris-base buffer (pH 8.3). Reactions were run in duplicate for 4 hours at 37°C in the dark in a flat bottom OptiPlate 96

well microplate (Perkin Elmer, cat. No.6005290). Fluorescence was measured using a Multi-detection Microplate Reader (SynergyTM HT, BioTek Instruments) at respective excitation and emission wavelengths of 360nm and 460nm. The coefficient of variation of repeated measures was 9%. The specificity of ACE-mediated substrate cleavage was monitored in control reactions where 1µM Lisinopril (Ratiopharm, Madrid-Spain) was added. This was compared to signal of control incubations with 7.5µg ml-1 of purified *ACE* enzyme (rabbit-lung, Sigma, cat. no. A-6778) in the presence or absence of Lisinopril 1 µM. Under these conditions 96% and 75% of substrate conversion by the purified enzyme and total homogenate, respectively, was blocked with Lisinopril. The signal was calibrated versus the emission of o-amino-benzoylglycine (Abz-Gly; Bachem cat. no. E-2920) as titrated between concentrations of 1.5 and 20 µM.

Immunohistological detection of Tenascin-C protein

Cryosections were prepared from vastus lateralis muscle, and subjected to immunological staining for *Tenascin-C* using the MA3 antibody essentially as previously described (16). Nuclei were counterstained with hematoxylin.

Statistics

All statistical analyses were performed using Statistica 10 (Statsoft, Tulsa, USA). Changes between the pre- and post-training findings were analyzed with a repeated ANOVA for the factor "genotype rs2104772" (A/A, A/T, or T/T) and the repeated factor "training" (yes or no). Fisher's post-hoc test was chosen based on whether data were normally distributed. To compare training-induced changes between parameters, values were normalized to the pre-training values prior to the ANOVA. Compliance with Hardy-Weinberg equilibrium was assessed using an online calculator, i.e., http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html. Post- vs. pre-exercise changes in *Tenascin*http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html. Post- vs. pre-exercise changes in -C expression were assessed using a paired T-test. Relationships were calculated based on Spearmans rank correlations and called significant at $p<0.05$.

RESULTS

Effect of training state on Tenascin-C transcript expression after single endurance exercise

Table 1 presents the anthropometric data for the subjects of group 1. We assessed *Tenascin-C* expression in vastus lateralis muscle after one-legged exercise in the first 8 hours after exercise because a robust response was reported for this time frame (Busso et al. 2013, Egan et al. 2013, Schmutz et al. 2010). The characteristics of the exercise stimulus are shown in Fig. 1. *Tenascin-C* transcript levels were increased eight hours after endurance exercise in trained, but unaltered in untrained (p=0.92), subjects (Fig. 2A). *Tenascin-C* transcript levels were neither altered three hours after endurance exercise in the untrained subjects (Fig. 2B).

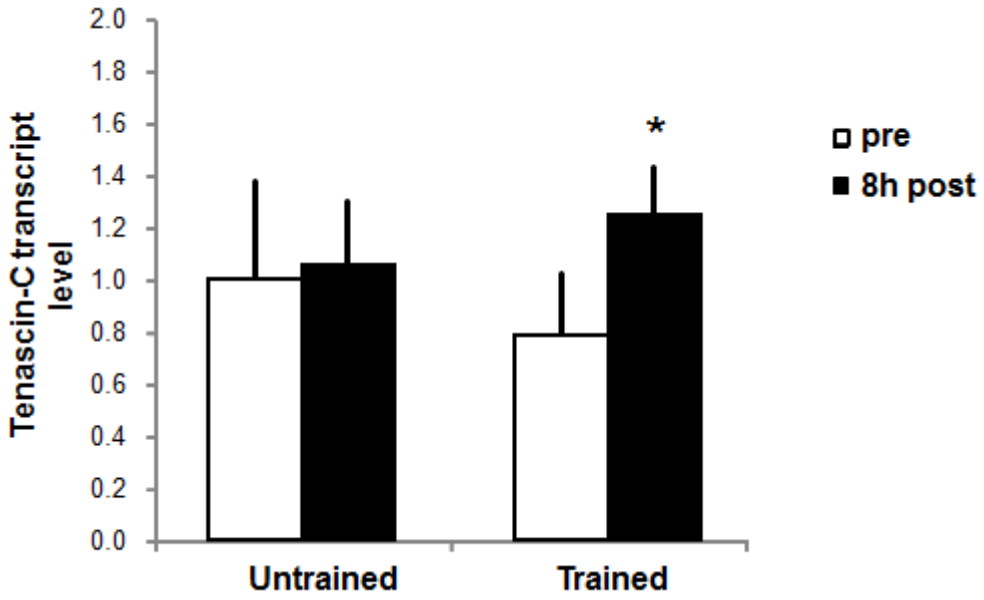
Effect of ACE inhibition on Tenascin-C/VEGF transcript expression after exercise

Intake of the *ACE* inhibitor Lisinopril produced an increase in *Tenascin-C* transcript levels in vastus lateralis of untrained subjects three hours after exercise (Fig. 2B). Similarly, *VEGF*-A transcript levels were specifically increased 3 hours after exercise under Lisinopril intake (Fig. 2C). Level alterations in *Tenascin-C* and *VEGF* transcript with exercise demonstrated an interaction effect with Lisinopril treatment (*Tenascin-C*: P=0.09, *VEGF*: P=0.04).

Table 1: *Performance characteristics of subjects of group 1* (mean ± SE). * denotes P<0.05 vs. untrained subjects without Lisinopril.

parameter	*untrained subjects without lisinopril* (n=7)	*untrained subjects with lisinopril* (n=7)	*trained subjects* (n=7)
Age [years]	23.1 ± 1.5	33.7 ± 3.2	27.1 ± 2.6
Weight [kg]	75.1 ± 4.8	76.3 ± 5.7	80.9 ± 4.1
Height [cm]	178.2 ± 3.0	175.3 ± 4.2	182.9 ± 2.1
VO2 max 2-leg [ml O2.min-1.kg-1]	47.6 ± 1.5	49.3 ± 2.5	60.2 ± 2.6 *
Pmax 2-leg [W]	270.0 ± 13.5	295 ± 15.1	370.0 ± 15.6*
VO2 max 1-leg [mL O2.min-1.kg-1]	40.6 ± 1.8	44.6 ± 4.7	46.5 ± 1.8 *
Pmax 1-leg [W]	169.3 ± 9.9	174 ± 10.9	233.6 ± 9.7 *

2a)



2b)

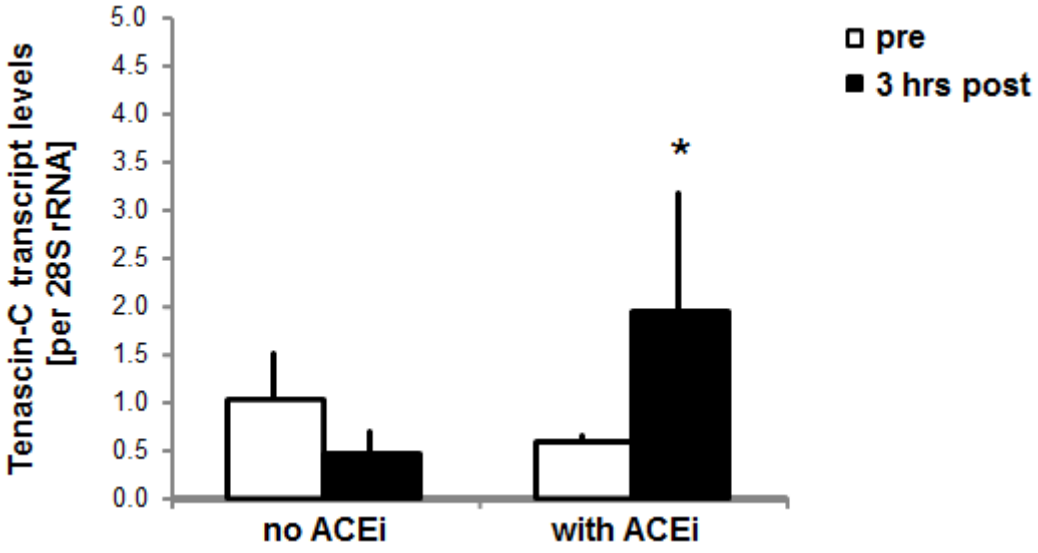


Figure 2. *Tenascin-C* | *VEGF*| *VEGF*-A transcript levels after one-legged exercise. (*a, top*) Bar graph visualizing the mean + SE of *Tenascin-C* transcript levels in trained and untrained subjects 8 hours after one-legged exercise (*n* = 7). *Tenascin-C* (b, bottom) and *VEGF*-A (c, page 108) transcript levels 3 hours post-exercise in untrained subjects with or without consumption of the *ACE* inhibitor Lisinopril (*n* = 7 each group). **P* < 0.05 vs. pre-exercise values (paired T-test).

2c)

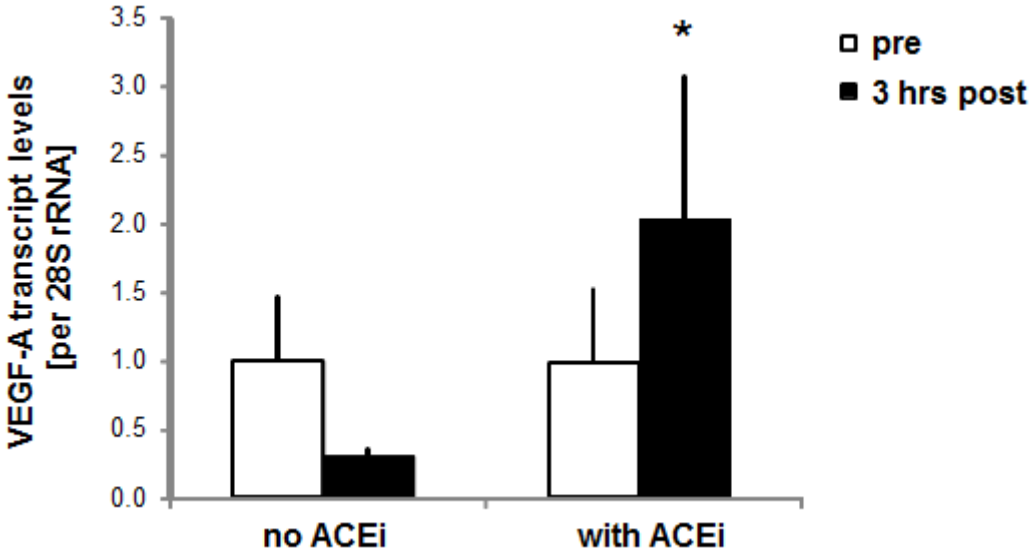


Figure 2c. *For description of figure 2a-c see page 107*

Effect of exercise and ACE inhibition on ACE activity and angiotensin 2 levels

Mean serum concentration of angiotensin 2 increased 1.6-fold after the bout of one-leg endurance exercise in untrained, but not trained subjects (p=0.04 vs 0.34; table 2). Mean serum angiotensin 2 concentrations also increased 1.4-fold after endurance exercise in untrained subjects that consumed Lisinopril. This increase did not differ to subjects that did not consume Lisinopril (P = 0.682). *ACE* activity in vastus lateralis muscle before exercise was higher in untrained subjects that consumed Lisinopril compared to untrained and trained subjects that did not consume Lisinopril (table 1). Blood pressure was not affected by Lisinopril consumption (table 2). Fold changes in serum angiotensin 2 concentrations after exercise were negatively correlated to muscle *ACE* activity at baseline (rho=-0.75).

Table 2: *Angiotensin related parameters of group 1* (mean ± SE). * denotes P<0.05 vs. untrained subjects without Lisinopril. $ denotes P<0.05 vs. values before exercise.

	untrained subjects without lisinopril	*untrained subjects with lisinopril*	*trained subjects*
Blood pressure at rest			
Systolic [mm Hg]	117.3 ± 2.6	128.8 ± 5.6	120.5 ± 2.5
Diastolic [mm Hg]	69.7 ± 2.9	71.6 ± 4.0	73.2 ± 1.7
Blood pressure at rest after 3 days Lisinopril			
Systolic [mm Hg]		129.4 ± 4.3	
Diastolic [mm Hg]		66.2 ± 4.0	
ACE activity [pmol min-1 mg-1 protein]			
before exercise	36.5 ± 5.9	67.4 ± 13.5 *	37.0 ± 6.4
Angiotensin 2 [pg mL-1]			
before exercise	18.4 ± 3.3	17.4 ± 7.1	17.6 ± 7.9
after exercise	29.9 ± 7.9 $	25.0 ± 10.2 $	40.8 ± 18.2

Effects of endurance training

In group 2, we assessed the effect of endurance training on *Tenascin-C* protein expression. Table 3 reports the pre-training biometric data of the studied subjects. Endurance training on the bicycle ergometer improved $\dot{V}O_2max$ (+9%) and local components of aerobic fitness in the vastus lateralis muscle, including the capillary-to-fiber ratio (+13%), capillary density (+10%), and mitochondrial density (+24%; table 3).

Association of Tenascin-C expression with training

Tenascin-C was present in the vastus lateralis muscle in both 250-kDa and 200-kDa isoforms (Fig. 3). Endurance training increased the concentrations of both the large (77 ± 29% increase) and small (128 ± 54% increase) isoforms. *Tenascin-C* staining was associated with capillary structures and blood vessels in the interstitium (Fig. 3C-E).

Table 3: *Biometric characteristics of subjects of group 2* (mean ± SE)

parameters	*before Training* (n=36)
Age [years]	32.9 ± 1.6
Weight [kg]	77.4 ± 2.3
Height [cm]	177.6 ± 1.2

3a)

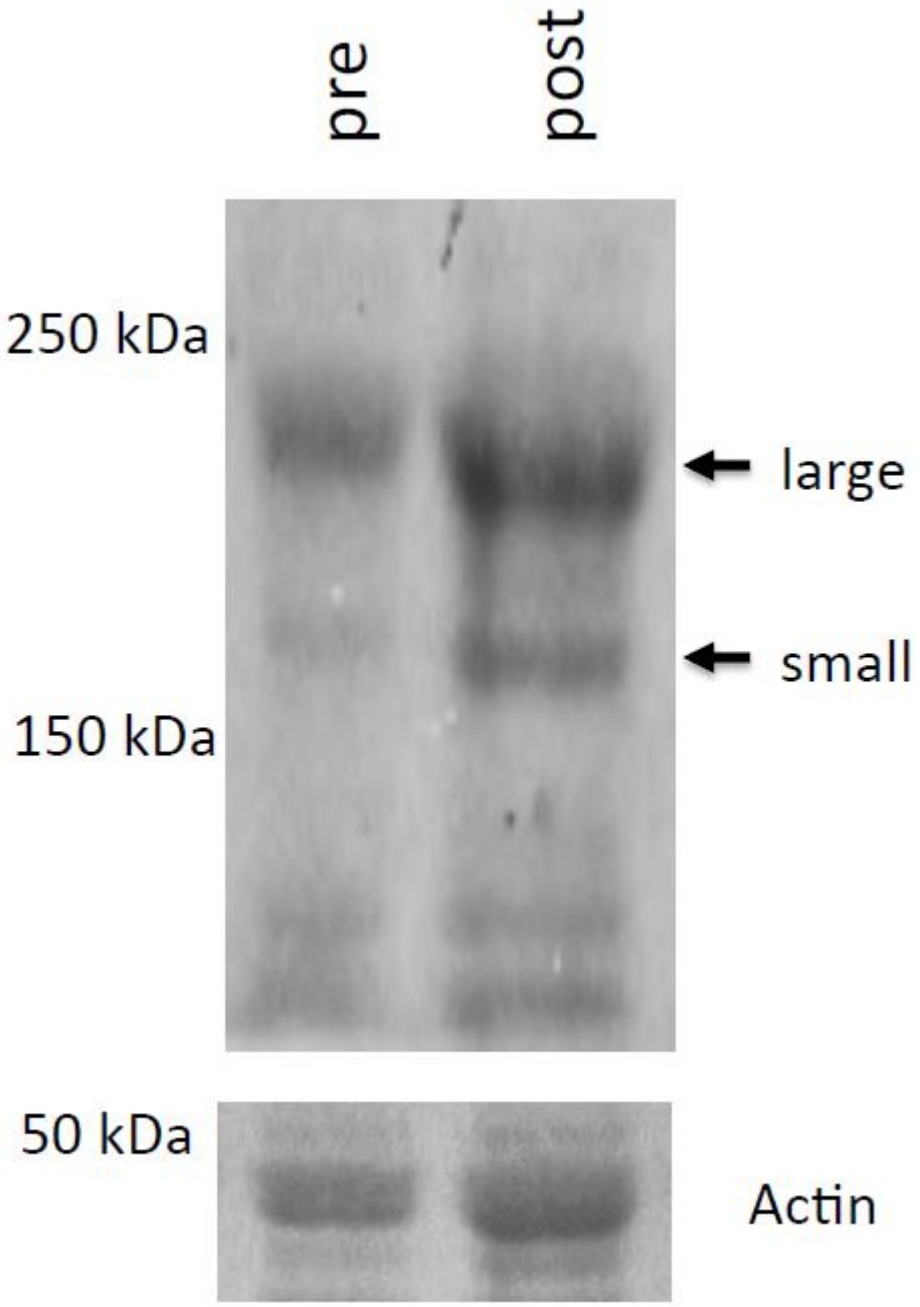


Figure 3a-e. *Tenascin-C* protein isoforms in vastus lateralis with endurance training. (*a, above*) Immunoblot showing the small (180 kDa) and large (230 kDa) *Tenascin-C* isoforms in one subject before and after endurance training. The position of the actin band (loading control) on the Ponceau S-stained membrane before immunoblotting is indicated. (*b, page 114*) Bar graph of mean + SE of the fold changes in *Tenascin-C* expression. **P* < 0.05 vs. pre. ANOVA with Fisher's post-hoc test (*n* = 17). (*c, page, 117; d, page 116*) *Tenascin-C* staining (orange staining) in the vastus lateralis muscle of one subject before (*c, page 115*) and after (*d, page 116*) endurance training and in another subject before training (page, 119). Nuclei are stained in blue. Arrows and arrowheads point to *Tenascin-C* staining in capillary structures and blood vessels. Bar = 100 µm.

3b)

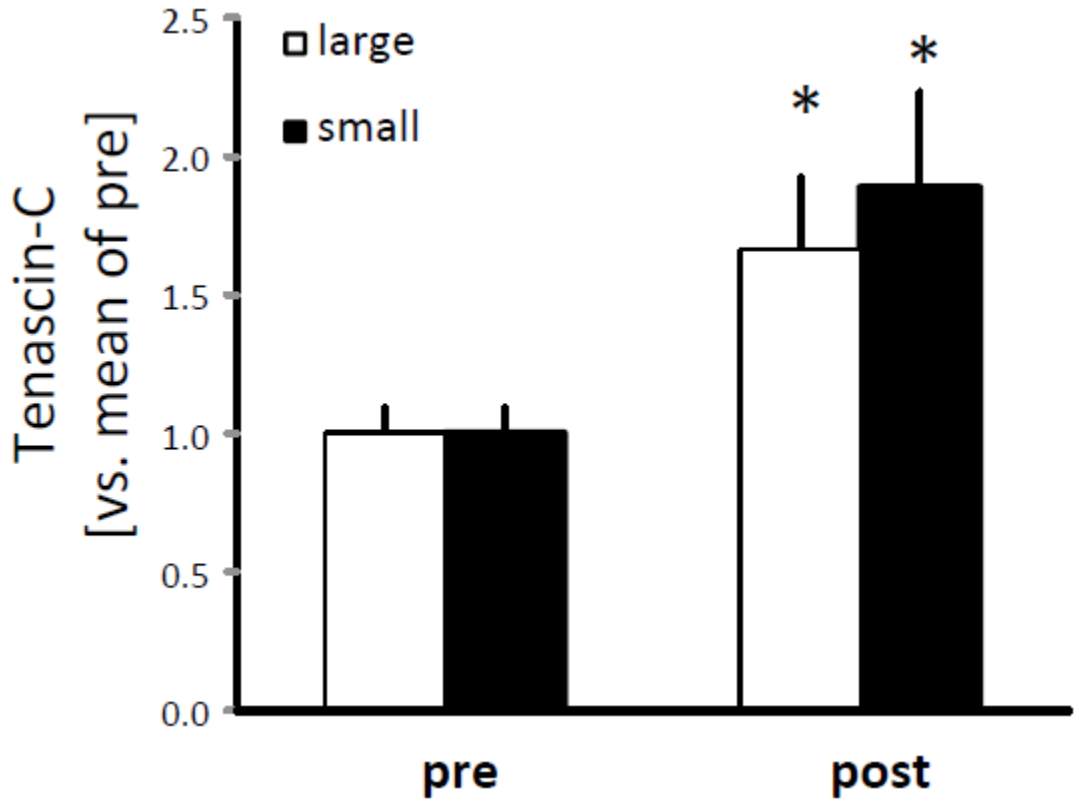


Figure 3b. *description for figure 3 see page 113*

3c)

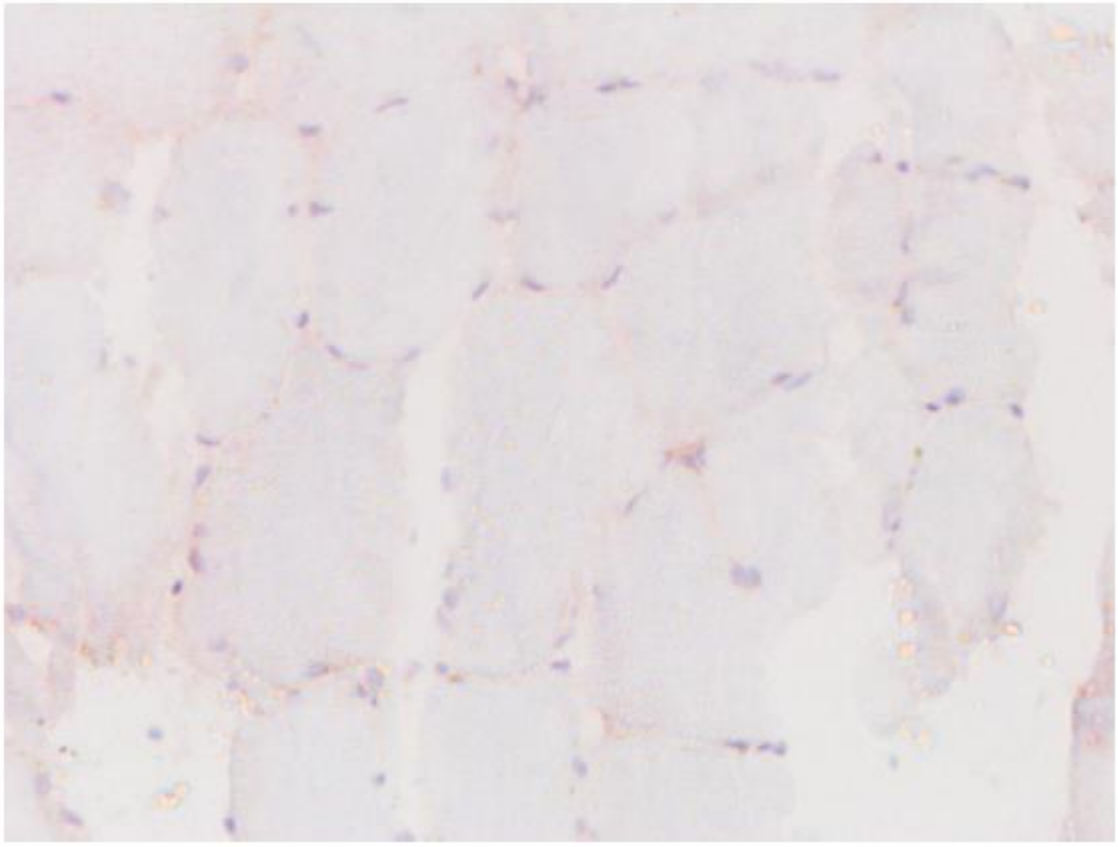


Figure 3b. *description for figure 3 see page 113*

3d)

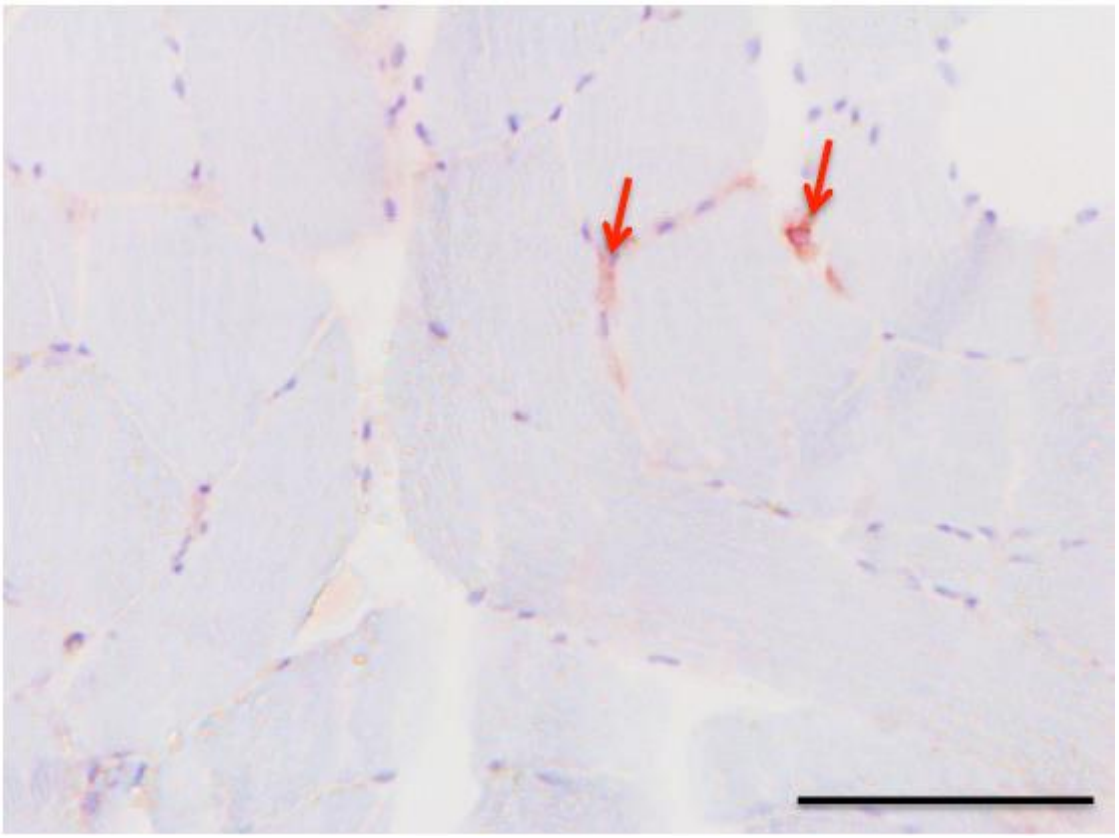


Figure 3d. *description for figure 3 see page 113*

3e)

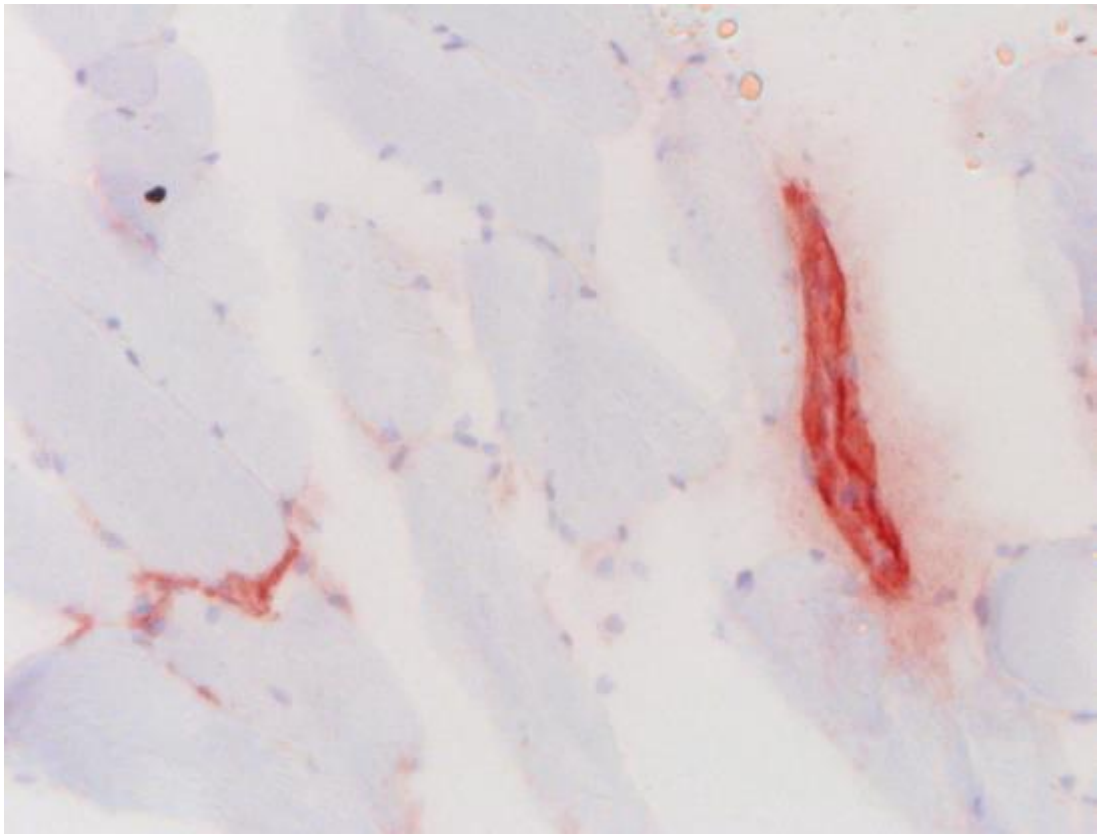


***Figure 3e**. description for figure 3 see page 113*

SNP rs2104772 within the Tenascin-C gene affects training-induced gains in muscle capillary-to-fiber ratio

With regard to several muscle parameters, we assessed the influence of the *Tenascin-C* SNP rs2104772, which is characterized by a thymidine-to-adenosine exchange at nucleotide position 44,513. Fig. 4 presents an example of the genotyping reaction. Statistical analysis revealed that we could not reject the assumption that the study population was in a Hardy-Weinberg equilibrium (P = 0.0854, n=36).

The baseline data showed that the small *Tenascin-C* isoform tended to be present in 28% lower amounts among individuals with the T/T variant compared to those with A/T (p=0.045). Endurance training improved aerobic function and produced adjustments of associated structures in vastus lateralis muscle, i.e. capillaries, mitochondria and myofibrils (table 4). Three of these parameters demonstrated an interaction effect between endurance training x polymorphism rs140772 (P < 0.01, table 3). Figure 5 resolves the values for the three respective genotypes of polymorphism *rs2140772*. This concerned the volume density of central mitochondria per fiber, the volume density of total mitochondria per fiber and capillary-to-fiber ratio: Capillary-to-fiber ratio was increased in subjects with an 'A-allele' (i.e., the A/T and A/A genotypes of rs2104772), while this structural variable was reduced in those with the T/T genotype (−14%; fig. 5). Equally, the volume density of total mitochondria per fiber was increased in the A/T and A/A genotype after training and this distinguished to an unaltered value for this parameter in the homozygous T/T genotypes. The volume density of central mitochondria was 30%

increased only in association with the heterozygous genotype A/T but was not altered in those with the homozygous A/A and T/T genotypes. The fold changes in *Tenascin-C* protein with endurance training were not significantly associated with SNP rs2104772.

4a)

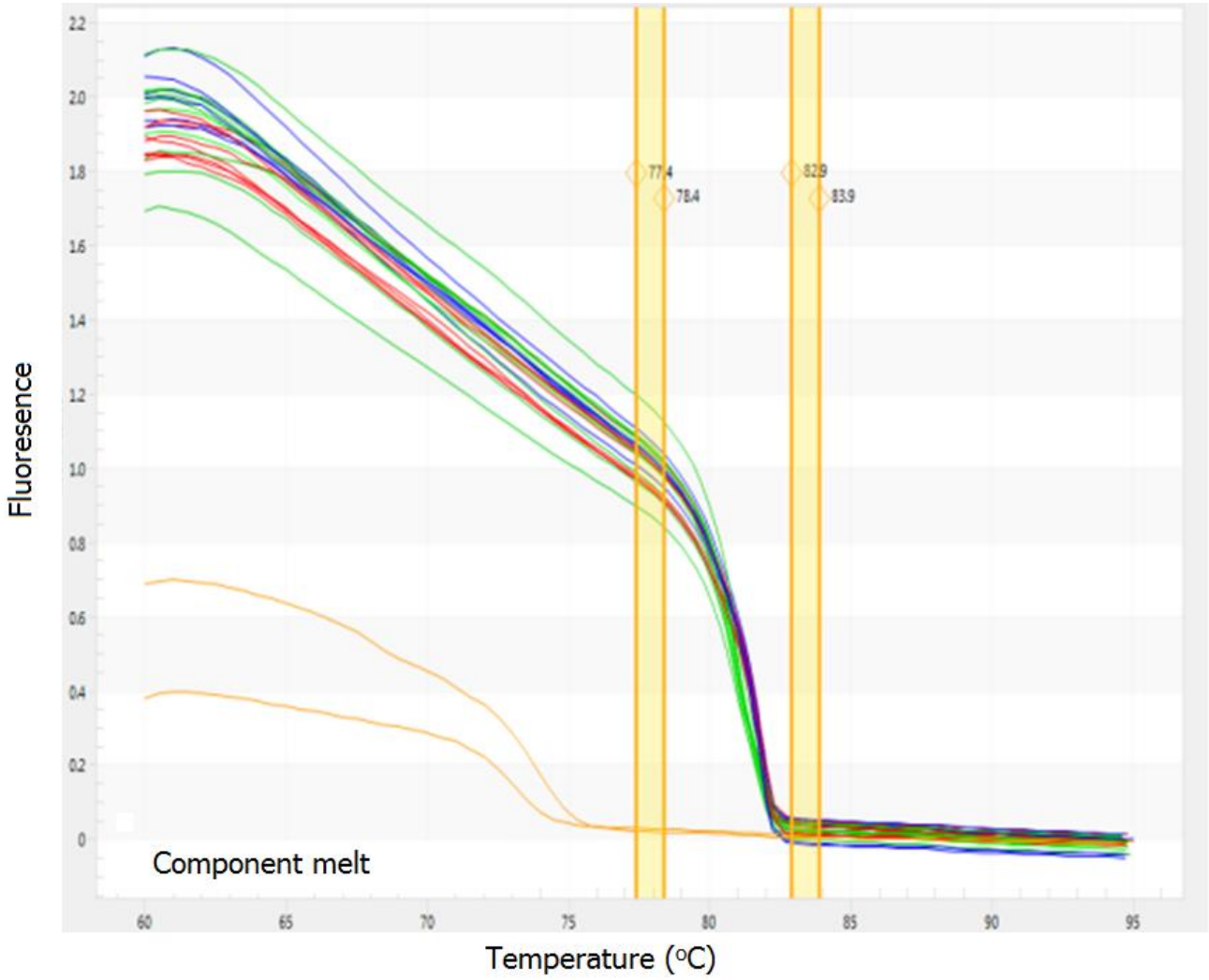


Figure 4a-f. Analysis of SNP rs2104772 by HRM. *(a, above; b, page 121; c, page 122)* Detected melting curves for the different genotypes, i.e., the homozygote A/A (red) and homozygote T/T (blue) relative to the heterozygote A/T (green). Every sample was analyzed in duplicate. (a, above) Raw data of the pre-melt, melt, and post-melt regions. (*b, page 121*) Normalized data derived from the raw data plots. (*c, page 122*) Curves derived from the normalized data with the A/A genotype set as a (horizontal) baseline. NTC, negative control (yellow). *(d, page 123; e, page 124; f, page 125)* Sequence analysis of the three identified genotypes in chromatograms presenting the forward sequence. Arrows link the single-nucleotide polymorphism (SNP) position 44,513. 'W' denotes heterozygosis for the SNP where a double-peak is present at position 44,513 for both sequenced alleles (nucleotides A and T simultaneously).

4b)

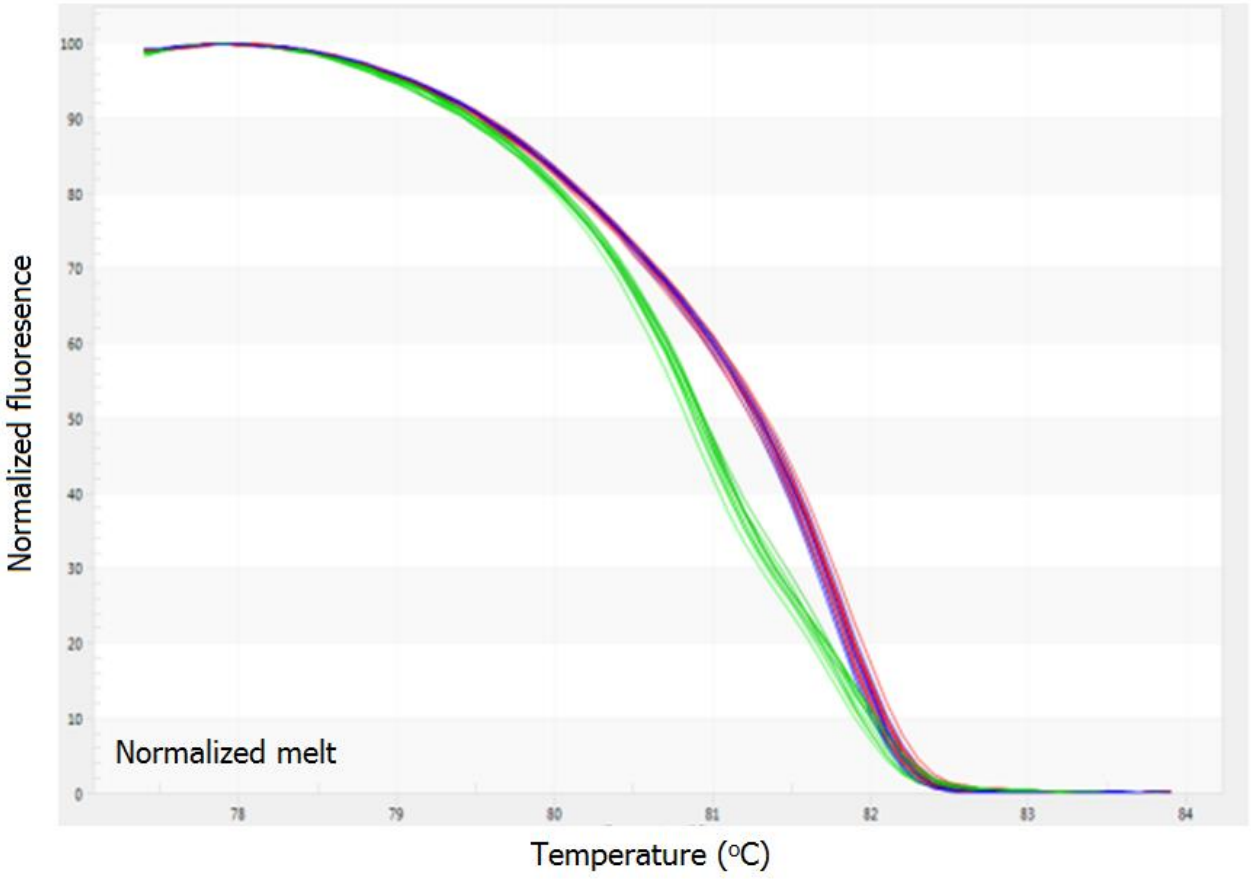


Figure 4b. *for a description of figure 4 see page 120*

4c)

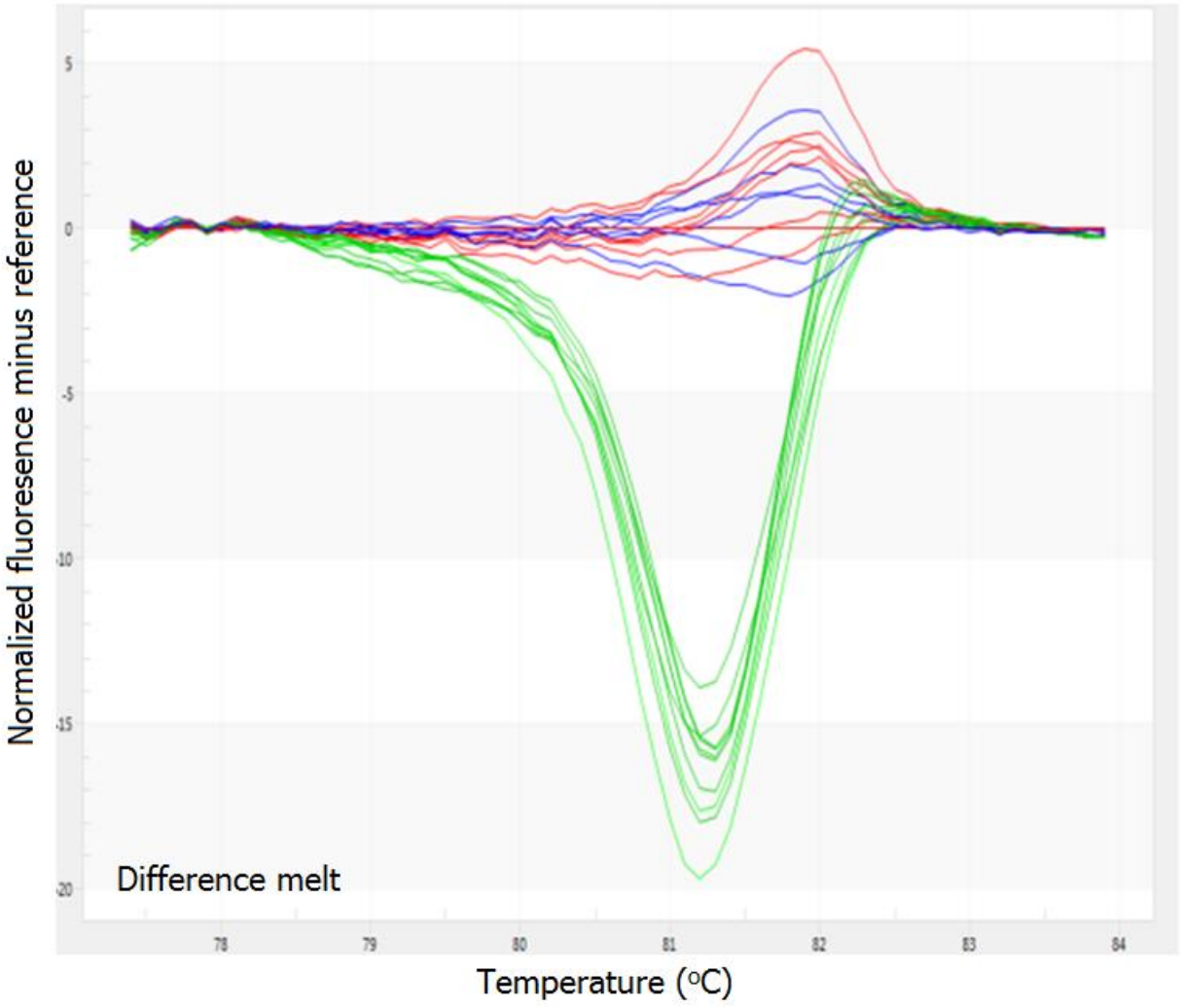


Figure 4c. *for a description of figure 4 see page 120*

4d)

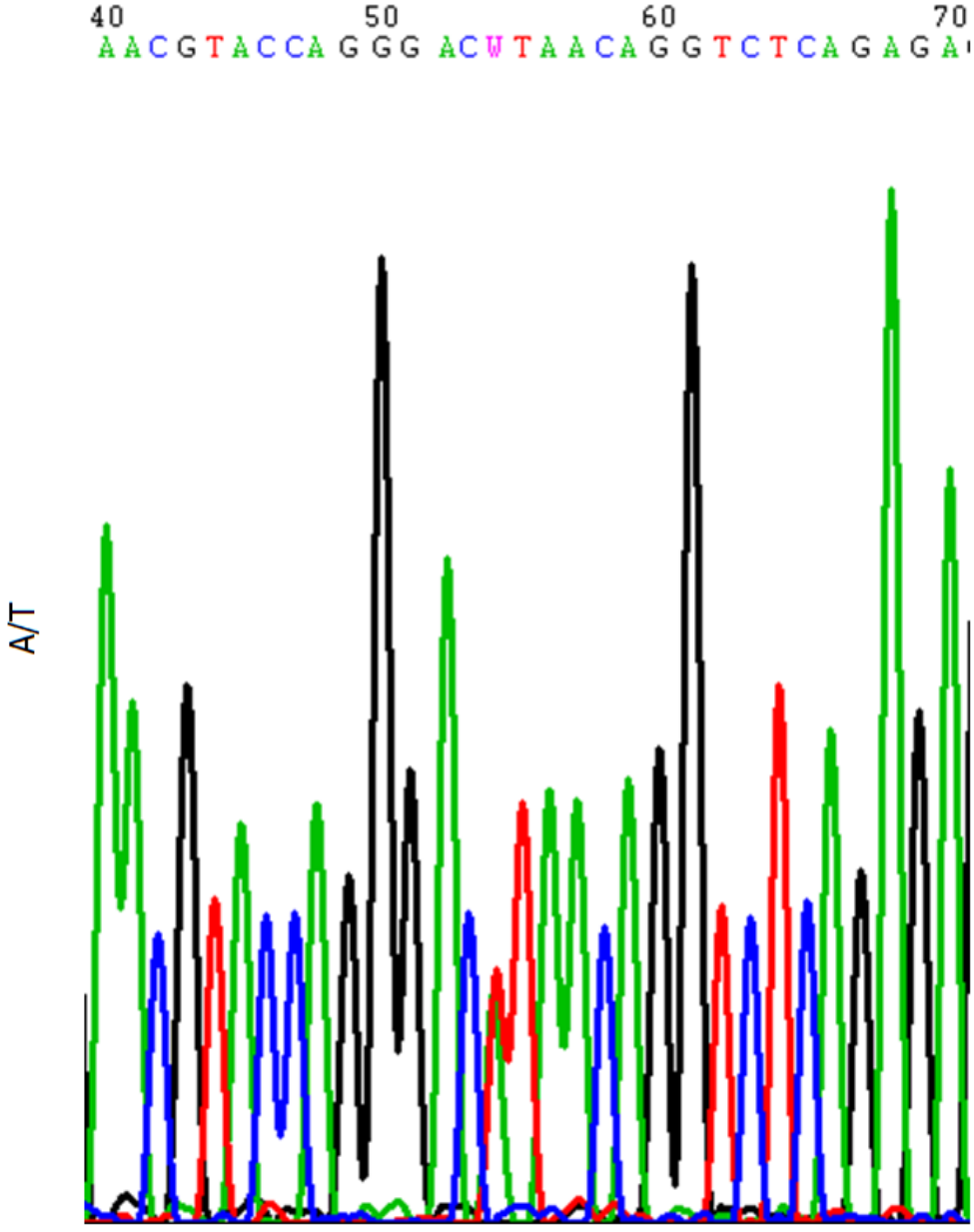


Figure 4d. *for a description of figure 4 see page 120*

4e)

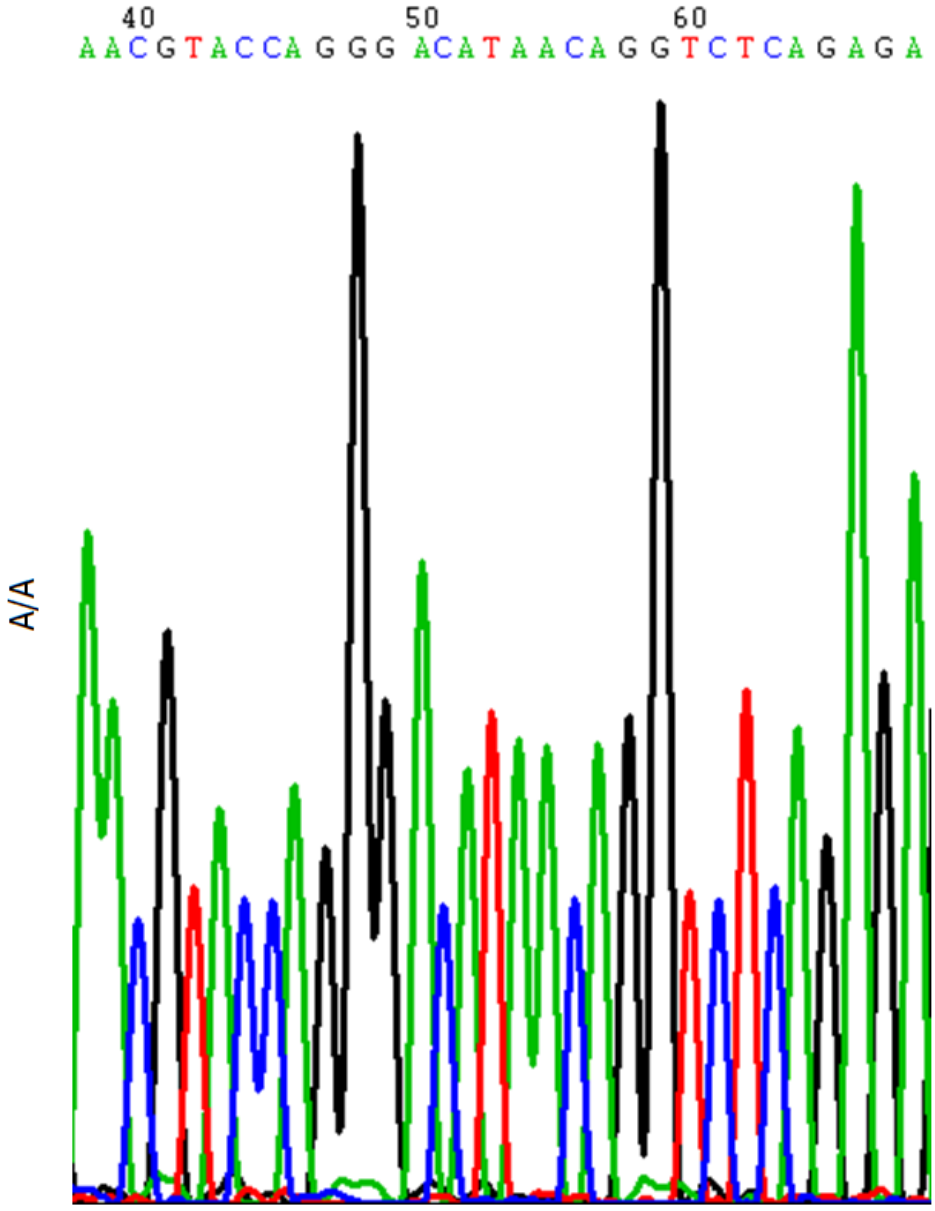


Figure 4e. *for a description of figure 4 see page 120*

4f)

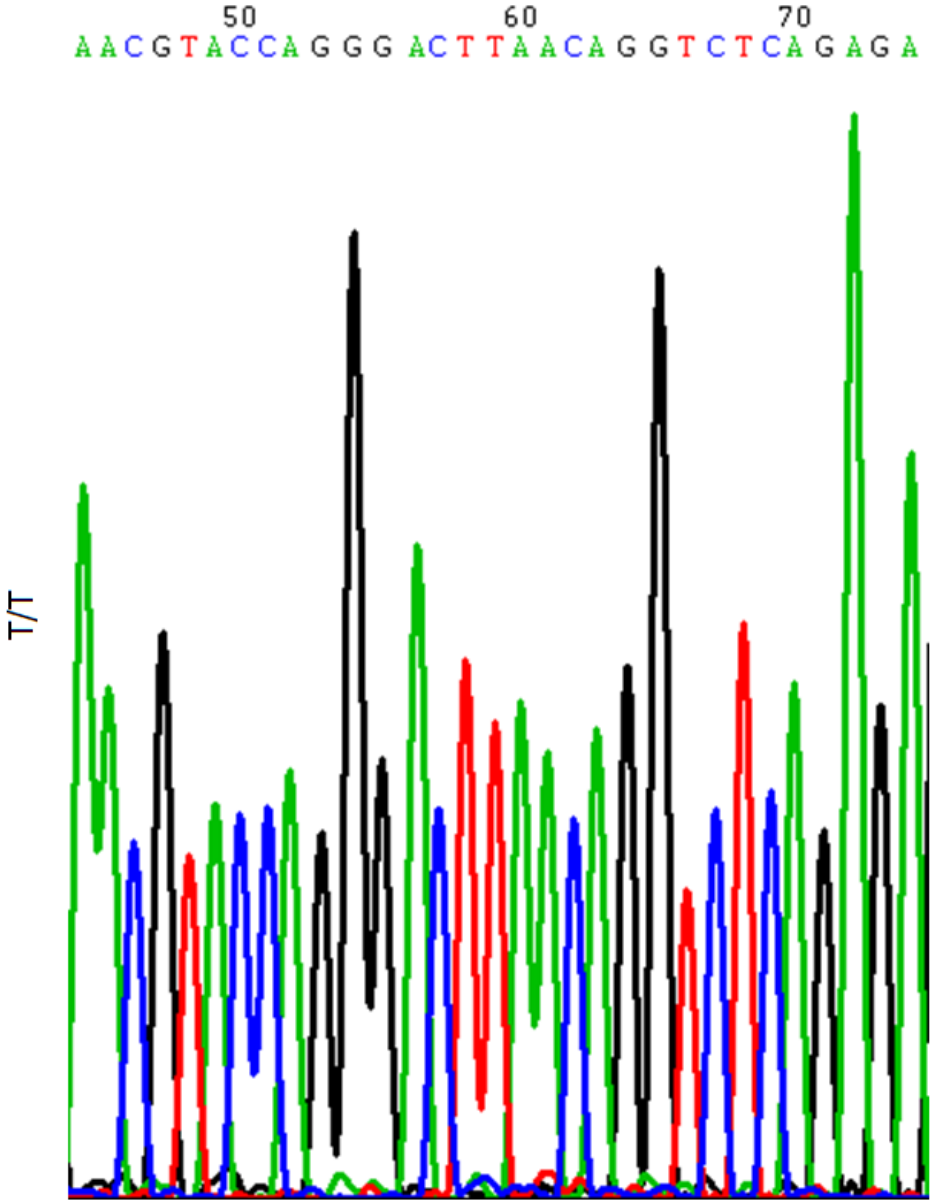


Figure 4f *for a description of figure 4 see page 120*

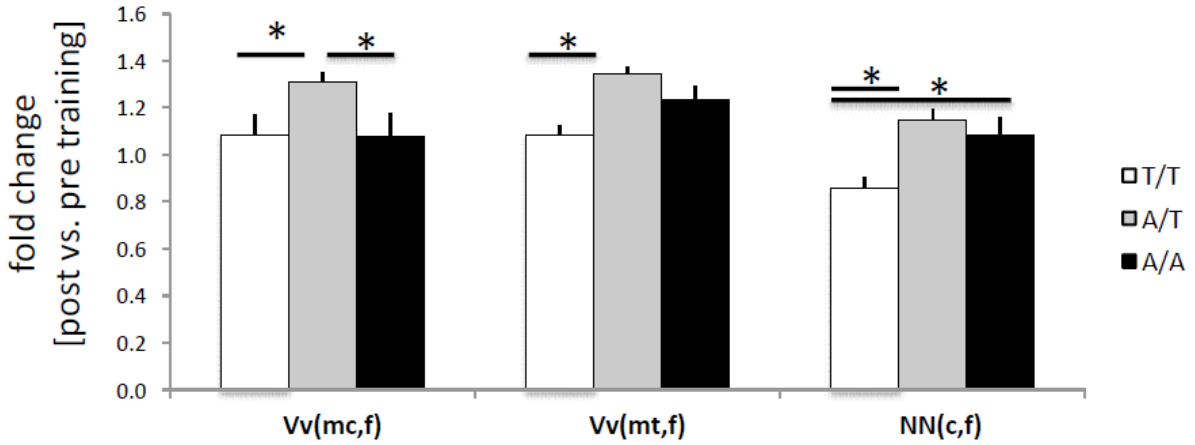


Figure 5. Genotype effect (rs140772) on the response of muscle variables to endurance training. Bar graph indicates the mean + SE of the fold changes in the three parameters of muscle energy supply that showed a genotype effect in regard to the response to endurance training. Fold changes are shown separately for the three genotypes of SNP rs140772 in the *Tenascin-C* gene—i.e., A/A (n = 8), A/T (n = 23), and T/T (n = 5). Abbreviations: Vv(mt,f), volume density of total mitochondria; Vv(mc,f), volume density of central mitochondria; NN(c,f), capillary-to-fiber ratio. *P < 0.05 for the indicated comparison, ANOVA with Fisher's post-hoc test.

DISCUSSION

Repeated blood flow increases are a major regulator of normal physiological angiogenesis in skeletal muscle (Egginton et al. 2001, Prior et al. 2004). The consequent increase in capillary density and capillary-to-fiber ratio conspires with exercise-induced vasodilatation to improve substrate supply to contracting skeletal muscle (Egginton et al. 2001, Mortensen et al. 2012, Prior et al. 2004). Flow-induced gains in muscle capillary density depend on the vasoconstrictor angiotensin 2 (Greene 1998). We previously demonstrated that genetic inhibition of angiotensin 2 production (through the presence of the I-allele within the *ACE* gene) exerts a negative influence on capillary density in vastus lateralis muscles of untrained subjects (Vaughan et al. 2013). In the current study, we assessed the associations of these effects with exercise-induced serum levels of angiotensin 2 and the expression of the pro-angiogenic extracellular matrix protein *Tenascin-C* (5), which has been suggested to induced by angiotensin 2 in smooth muscle cells during vascular remodeling in hypertension (Mackie et al. 1992). We hypothesized that endurance training-induced gains in capillarity are modulated by *ACE* activity and by the SNP rs2104772 within the *Tenascin-C* gene, which was predicted to affect adhesive function and the molecular interactions of this protein (Danser et al. 2007, Rigat et al. 1990) (Fig. 6).

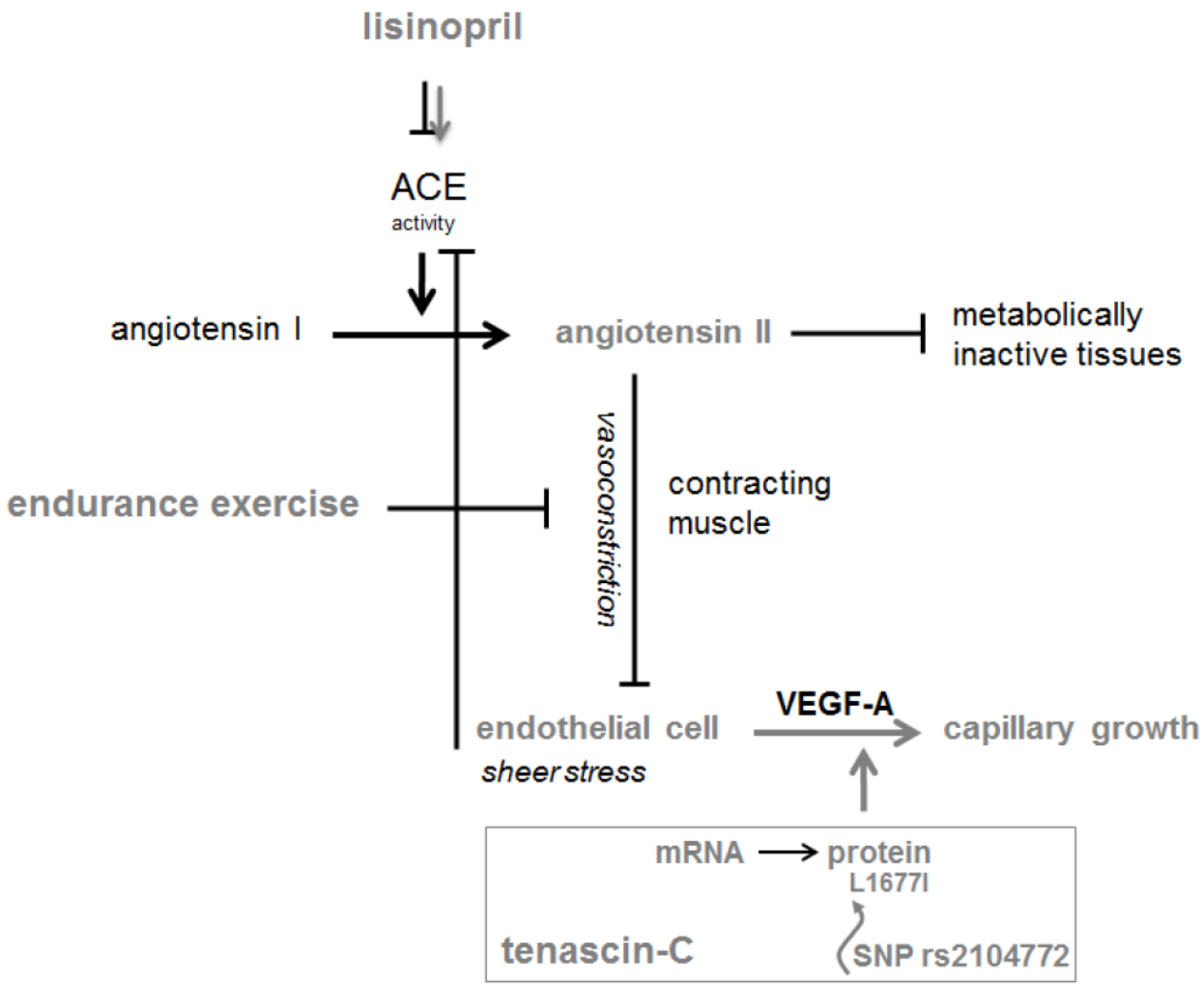


Figure 6. Interactions between exercise-induced responses in serum angiotensin and *Tenascin-C* and with regard to angiogenesis. Lines with arrows of a hyphen at the end, respectively, indicate promotion or inhibition of the connected process. A curved line indicates a qualitative effect. Grey lines represent the interactions identified in this investigation.

Obviously, certain limitations apply when drawing mechanistic conclusions from a sport-medical intervention in humans which involves genetic analyses. The polymorphism within the *Tenascin-C* gene was studied in subjects with different genetic backgrounds, although they shared the same ethnicity. Additionally, the number of subjects included in the study population was low compared to that in many genetic studies. However, due to the invasive nature of the study (requiring an otherwise unnecessary muscle biopsy), we were ethically bound to use as few subjects as possible. In order to avoid that a potential regulation of transcript expression would have been missed, the sample points for our study were set to times in the window between 3-12 hours after exercise when the major expression response in vastus lateralis muscle during recovery from two-legged bicycle ergometer exercise occurs (Busso et al. 2013, Egan et al. 2013, Schmutz et al. 2010). Despite these considerations we were unable to assess the effect of single leg exercise and Lisinopril 3 hours after the exercise in trained subjects. Furthermore, the present comparison of the effects of two different exercise types (i.e., one-legged and two-legged exercise) poses a potential limitation for the interpretation. The one-legged type of exercise was selected to increase the metabolic strain on the exercised and tested muscle— maximizing the stimulus for recruited muscle group, while reducing the effects of central restraints on exercise performance (McPhee et al. 2009). While supporting a role of *ACE* for the expression of the pro-angiogenic factors *Tenascin-C* and *VEGF*-A in untrained muscle after exercise, the underlying mechanism, and its involvement in training-induced angiogenesis awaits further exploration. In this regard, we identify that studying the influence of polymorphism rs2104772 on training-induced adjustments in muscle

composition provides a valid approach to probe the implication of *Tenascin-C* in capillary remodeling.

The identified effects of rs2104772 on gains in capillary density and capillary-to-fiber ratio with endurance training (Fig. 5), support the notion that *Tenascin-C* is actively involved in the processes that instruct exercise-induced capillary growth. By contrast, other structural parameters related to myofibrils were not affected (table 4). In fact, increases in the capillary-to-fiber ratio alone reportedly reflect effective capillary remodeling, as volume density alterations often mirror volumetric changes in muscle fiber cross-section (Egginton et al. 1998). This suggests that the function of *Tenascin-C* is associated with the activity-induced structural rearrangement of capillaries with respect to muscle fibers.

Our present molecular characterization focused on the observed modulation of endurance training-induced muscle capillarity by SNP rs2104772, which exists within the coding region of the *Tenascin-C* gene. The T-to-A exchange at nucleotide position 44,513 leads to the substitution of isoleucine instead of leucine at amino acid position 1677 in the C-terminal FNIII domain of TNC (Matsuda et al. 2005). Based on structural modelling this domain was predicted to recognize the 13th fibronectin type III domain of fibronectin, and therefore this SNP interferes with fibrinogenesis and wound healing (Chiquet et al. 2003). The structural model predicts that the presence of isoleucine rather than leucine causes beta-sheet instability and may negatively affect *Tenascin-C* molecular elasticity (Matsuda et al. 2005, Saunders et al. 2013), thus reducing the capacity of *Tenascin-C* to dissolve cell–matrix contacts. While experimental proof for this molecular interaction is

missing, the in here identified effects of rs2107772 on muscle capillarity and the association of rs2107772 with the Achilles tendon rupture and asthma provide a further indication for the functional relevance of the resultant amino acid exchange for Tenascin's interactions within the extracellular matrix (Matsuda et al. 2005, Saunders et al. 2013). Hence, the observed larger increase in capillary density associated with the A/A and A/T genotypes as opposed to the T/T genotypes are intriguing. This ambivalence suggests that a reduced elasticity and altered functional interaction of *Tenascin-C* with its extracellular partners are potential parameters to explore the biophysical foundation that underlies the superior gains in muscle capillarity in A/A and A/T genotypes of SNP rs2107772.

The latter observation is also of interest because subjects heterozygous for SNP rs2104772 showed the largest increase in central mitochondria after endurance training (Fig. 5). This suggests the possibility that this observation may result from an equilibrium of two opposing processes: the interaction of an optimal condition for mitochondrial biogenesis with endurance exercise in the A/A variant, offset by respiratory problems associated with the A/A genotype (Matsuda et al. 2005, Saunders et al. 2013). We cannot presently directly address this interpretative hypothesis, as differences in oxygenation or ventilation were not assessed in the current study.

Increased *Tenascin-C* expression promotes neovascularization, and its enhanced expression by vascular smooth muscle cells may mediate angiotensin II-induced changes in vascular structure during hypertension (Ballard et al. 2006, Mackie et al. 1992). Both endothelial and smooth muscle cells are potential sites of

Tenascin-C expression and action (Ballard et al. 2006, Intengan et al. 2000, Vollmer et al. 1990). The presently observed *Tenascin-C* expression in the studied muscle group (Fig. 3C–E) is consistent with an expression in the capillary endothel. Smooth muscle cells are mainly found in the arterioles of skeletal muscle, which integrate exercise-modulated control of muscle perfusion through inhibition of angiotensin-mediated smooth muscle cell constriction/contraction via repeated contractions of the adjacent muscle fibers (Clifford et al. 2004, Wunsch et al. 2000). In this regard, it was puzzling that the expression responses of the small and large *Tenascin-C* isoforms to endurance training were not significantly affected by the rs2104772 polymorphism (Fig. 3A/B). This suggests that the observed effect of rs2104772 may reflect a role of *Tenascin-C* in the stabilization of newly formed capillaries, rather than in promoting capillary growth. The present findings corroborate the view that *Tenascin-C* may have an important influence on the adaptations in mitochondria and capillaries that are typically connected with endurance training (Hoppeler et al. 1985).

An intriguing finding of our investigation was that *ACE* activity in vastus lateralis muscle was nearly two-fold higher in subjects which consumed Lisinopril and that this did not attenuate the rise in serum angiotensin 2 concentration post exercise (table 2). This observation relates to reports that *ACE* inhibitor intake (captopril) does not blunt the increase in angiotensin 2 post exercise (Aldigier et al. 1993), and that Lisinopril increases the renal and cardiac expression of *ACE* and ACE2, the latter of which counteracts *ACE* action (Jessup et al. 2006). As well we identified that blood pressure was not significantly affected by Lisinopril intake (table 2) although this is commonly observed with early treatment. Collectively, the

findings suggest that compensatory reactions of the angiotensin system were set in motion in skeletal muscle in consequence of the 3 days of Lisinopril consumption (reviewed in Avanzini et al. 2002). Thereby the negative correlation between fold changes in serum angiotensin 2 concentrations after exercise and muscle *ACE* activity highlights a possibly complex relationship of local and systemic renin-angiotensin system which may reflects the role of shear stress for endothelial *ACE* expression as well (Barauna et al. 2011).

An interesting aspect in this regard was the detection of an increased expression of the *Tenascin-C* transcript at three hours after one-legged exercise under Lisinopril consumption in untrained subjects but when the *Tenascin-C* transcript of untreated subjects was, unlike in trained subjects, not affected until eight hours post exercise (Fig. 2). This relationship possibly reflects differences in vasomotor reactivity and downstream muscle perfusion between trained and untrained subjects. With the onset of exercise, perfusion of skeletal muscle rapidly increases due to the inhibited contraction of smooth muscle cells in pre-capillary sphincters (Clifford et al. 2004). This vasodilatation is greater in trained subjects due to their elevated capillary density (Lithell et al. 1981) and exaggerated functional sympatholysis (Mortensen et al. 2012). The consequent hyperemia is a main contributor to the increased shear stress to capillary walls that, together with abluminal forms of stress, triggers angiogenesis in contracting skeletal muscle (Egginton et al. 2001). Our present findings bind *Tenascin-C* into this mechanism and suggest that exercise-induced *Tenascin-C* expression is established with repeated exercise. The observed interaction effects between exercise x Lisinopril treatment on *Tenascin-C* and *VEGF* transcript levels, do together with the higher *ACE*

activity levels in untrained subjects under Lisinopril treatment and post-exercise increases in serum angiotensin 2 concentrations indicate that expression of these pro-angiogenic transcripts in muscle depends on *ACE* activity. This observation is in line with the observation that an angiotensin II-related mechanism regulates *VEGF* during angiogenesis with chronic electrical stimulation of rat skeletal muscle (Amaral et al. 2001).

It is worth noting that we examined *Tenascin-C*-associated angiogenesis in the health-associated situation of exercise. This observation differs from that in studies assessing the role of *Tenascin-C* in vascular growth in pathological blood vessel remodeling as occurs in chronic angiotensin-mediated hypertension, implantation of a balloon catheter, or tumor growth (Egginton et al. 2001, Mackie et al. 1992, Majesky 1994, Mustafa et al. 2012). Such situations are characterized by chronic changes in cell composition and chronic inflammation, which deteriorate tissue function. Conversely, the increased capillarity with physical training is a part of quantitative changes in muscle composition that improve the energy supply to contracting muscle (Wibom et al. 1992, Zumstein et al. 1983).

Conclusion

Here we provide evidence for a role of *Tenascin-C* in exercise-induced capillary growth and mitochondrial biogenesis in the skeletal muscle of men. Further studies are needed to explore the precise cellular elements of the biological pathway underlying the observed plasticity of energy supply lines with repeated endurance exercise, which we find to include ACE-related *Tenascin-C* expression and to be modulated by SNP rs2104772 within the *Tenascin-C* gene.

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AUTHOR CONTRIBUTIONS

Author contributions: P.V., Sv.G., Ad.H, H.H., and M.F. conception and design of research; P.V., Sv.G., S.W., and M.F. performed experiments; P.V., Sv.G., S.W., and M.F. analyzed data; P.V., H.H., and M.F. interpreted results of experiments; Ad.H. and M.F. funding; P.V. and M.F. prepared figures; P.V. and M.F. drafted the manuscript; P.V., Sv.G., and M.F. edited and revised the manuscript

Experiments performed by Sv.G. (ACE inhibition group): Taking informed consent, height, weight, blood pressure, glucose, cycling ergometer tests, blood processing, AngII assessment, *Tenascin-C* and *VEGF* transcript levels measurements

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Chapter 5

Adjustments of Muscle Capillarity But not Mitochondrial Protein with Skiing in the Elderly

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Abstract

Downhill skiing in the elderly increases maximal oxygen uptake (VO_2max) and carbohydrate handling, and produces muscle hypertrophy. We hypothesized that adjustments of the cellular components of aerobic glucose combustion in knee extensor muscle, and cardiovascular adjustments, would increase in proportion to VO_2max.

Nineteen healthy elderly subjects (age 67.5 ± 2.9 years) who completed 28.5 days of guided downhill skiing over 3-months were assessed for anthropometric variables, cardiovascular parameters (heart rate, haematocrit), VO_2max and compared to controls (n=20). Biopsies of *vastus lateralis* muscle were analysed for capillary density and expression of respiratory chain markers (NDUFA9, SDHA, UQCRC1, ATP5A1) and the glucose transporter GLUT4. Statistical significance was assessed with a repeated ANOVA and Fisher's post hoc test at a p-value of 5%.

VO_2max increased selectively with ski training (+7±2%). Capillary density (+11±5%) and capillary-to-fibre ratio (12 ± 5%), but not the concentration of metabolic proteins, in *vastus lateralis* was increased after skiing. Cardiovascular parameters did not change. Fold changes in VO_2max and capillary-to-fibre ratio were correlated, and were under genetic control by polymorphisms of the regulator of vascular tone, ACE. The observations indicate that increased VO_2max after recreational downhill ski training is associated with improved capillarity in a mainly recruited muscle group.

Introduction

Downhill skiing is a popular outdoor activity. The muscular adjustments to ski training are poorly understood despite the considerable interest in this leisure time activity (Berg et al. 1995; Muller et al. 2011). It has recently been pointed out that 3 months of recreational downhill skiing positively affects physical fitness and psychological well-being in elderly subjects (Dela et al. 2011; Finkenzeller et al. 2011). Increased leg strength due to an enlarged cross section of slow type muscle fibres and improved whole body aerobic capacity, were identified as two important contributors to the effects of skiing on fitness (Dela, Niederseer 2011).

Aerobic metabolism appears to account for a larger portion of the energy demand during low intensity, downhill exercise on the slope (Scheiber et al. 2009) and gains in importance with increased training and skill level (Tesch et al. 1978). Due to the considerable mechanical requirements on extensor muscles, a large portion of muscle becomes activated during slow contractions (Berg & Eiken 1999); elevating glucose metabolism (Scheiber et al., Krautgasser et al. 2009; Tesch et al. 1978 , Larsson et al. 1978; Tesch 1995).

Three elements of the pathway which supplies environmental oxygen to working skeletal muscle, i.e. oxygen transport system, muscle capillarity and mitochondria, are known to set aerobic capacity (di Prampero 1985). As shown previously with other modalities of training, the repeated increase in energy expenditure with contractile work induces a compensatory response to cover the increased metabolic demand of contracting muscle (Baldwin 1996). Thereby it appears that the degree of plasticity at the local, i.e. muscle level, exceeds the one of central (i.e. cardiac)

mechanisms and oxygen transport capacity (Hoppeler et al. 1985). Regarding skeletal muscle, an increased capacity of at least two biochemical processes contribute to the improved aerobic conversion of blood-borne glucose in contracting skeletal muscle: Firstly, this may include elevated capillarity together with improved capacity for contraction- induced glucose uptake via the facilitative glucose transporter 4 (GLUT4; Dela et al. 1993). Secondly, prolonged exercise may produce an increase in mitochondrial volume density (Fluck & Hoppeler 2003), which we have shown previously is matched by maximal oxygen uptake (Kayar et al. 1994; Oosterhof et al. 2011).

We therefore hypothesized that Alpine skiing will increase the capacity for aerobic combustion of glucose in knee extensor muscles being recruited during skiing via an increase in capillary density and the concentration of mitochondrial protein in proportion with an improvement in VO_2max. This was tested by assessing changes in aerobic capacity in a group of healthy active elderly that attended a guided skiing intervention of 3-months duration and compared to a control group that refrained from skiing over the winter season. Capillary density and the content of enhancers for glucose uptake (i.e. GLUT4) and markers of mitochondrial respiratory complex I, II, III and V in the major knee extensor, *m. vastus lateralis* (Berg, Eiken 1995), were assessed in the skiing group before and after ski training. As adjustments to endurance exercise and elevated altitude have been shown to be affected by the insertion / deletion polymorphism (i.e. *ACE* I/D) in the gene for angiotensin converting enzyme (Vaughan et al. 2013; Woods et al. 2000) we assessed whether the response in elements of the pathway that sets aerobic capacity would be modified by the *ACE* I/D genotype.

Methods

Experimental design-A full description of the recruitment procedure, the design of the study and the participant's characteristics is given in the paper of Müller et al. (2011). In short, a group of 27 healthy subjects on a beginners or intermediate level of alpine skiing (13 females (age: 67.2 ± 0.8 years; mean ± SE) and 14 males (age: 67.7 ± 0.8 years) started a 12-week skiing intervention. In parallel, a control group, consisting of 10 female subjects (age: 66.3 ±1.2 years) and 10 male subjects (age: 68.2 ± 1.6 years) was recruited and asked to live their normal life but they were not allowed to ski during the study. Baseline measurements, including the collection of a muscle biopsy in the skiing group, were carried out in an entry test before the intervention and the same measurements were repeated after the 12 weeks of skiing. Due to various reasons 5 subjects (i.e. 3 females, 2 males) dropped out during the intervention (Niederseer et al. 2011). The Ethics Committee of the University of Salzburg, Austria, approved the study. Informed consent was obtained from all participants before testing. The study thus meets the ethical standards of the journal.

Skiing protocol- 19 subjects completed the 12-week skiing intervention which consisted of a total of 28.5 ± 0.6 days of on average 3.5 hours of guided skiing in the Salzburg Ski Amadé resort (altitude 728-2700 m above sea level). Subjects were divided into four homogeneous performance groups according to their skiing skills on the first day. On average they descended 4885 meter during nine runs on the slope (6.6 minutes per run) per day.

Entry and exit test- Weighing and measuring of the subjects was conducted using high precision tools (Seca 709 and Seca 224, Seca, Hamburg, Germany) and fat mass was assessed as previously published using a calliper in 4 standardized points as described (Niederseer et al. 2011). Erythrocyte content was measured in a venous blood sample that was drawn from the cubital vein of the subjects (Narici et al. 2011; Niederseer et al. 2011). Muscle thickness before and after training was determined using a digital ultra-sonographer (MyLab25, Esaote, Genoa, Italy) with the subjects lying supine on a gurney. Scans were acquired at 50% of muscle length, mid-belly, in the mid-sagittal plane using a 7–10MHz linear-array probe. Care was taken to assure the scanning of the same anatomical location pre and post training by positioning the ultrasound probe in the mid-sagittal plane, orthogonal to the mediolateral axis, versus reference points that were collected by marking the probe position on acetate paper using moles and small angiomas. Muscle thickness was defined as the distance between the deep and the superficial aponeuroses. Muscle thickness was estimated using the public domain NIH software "ImageJ" (version 1.42q, National Institute of Health, USA, http://rsb.info.nih.gov/ij) from the average of three measurements along the aponeurosis. Aerobic exercise capacity was also assessed before and after ski training in an incremental performance test on a stationary cycle ergometer as described elsewhere (Muller et al. 2011). Maximal aerobic performance (Pmax) and maximal oxygen uptake (VO_2max) were determined using ergospirometry. Due to technical inaccuracy of data sampling, VO_2max values at baseline were excluded in three subjects from further analysis. Heart rate (HR) was registered by continuous ECG and blood pressure was measured every two minutes during ergometry. During ski training HR was

monitored by Suunto watches and heart rate belts (t6, Suunto, Helsinki, Finland). A biopsy was collected from *vastus lateralis* muscle of the left leg prior to and 5-7 days after ski training with a Weil-Blakely conchotome (Gebrueder Zepf Surgical Instruments, Dürbheim, Germany), snap frozen and stored at -80°C.

Muscle histology - Frozen biopsy samples were mounted with Tissue-Tek® OCT TM on cork and 15-µm cross-sections were prepared using a cryostat (Leica CM 1800) by adjusting the cutting plane in perpendicular direction to the major axis of the muscle fibres. Sections from pairs of pre/post samples were mounted on Superfrost slides (Menzel, Germany), dried at room temperature and stored in sealed tubes at -80°C. Slides were stained using a lectin antibody as described by Ahmed et al. (1997). In brief, slides were thawed, fixed with acetone for 15 min, blocked with 0.1 % BSA in HEPES for 60 min, incubated with a peroxide solution for 30 min and incubated with the lectin antibody (Ulex Europeus 50 µg/ml in 1 % BSA in HEPES). Between every step, a 5 min wash with HEPES was conducted. For the colour reaction, the Vectastain ABC and DAB kit (Vector Laboratories, Peterborough, UK) were used. Slides were finally embedded in glycerol gelatine (Sigma) and covered with cover slips.

Quality of the staining was inspected and signal was recorded at 20x magnification against the scale of a 1-mm-graticle with an Axiocam MRc camera that was mounted on a microscope (Zeiss Axioskop 2 mot plus). The different images of one section were combined in one final image using Microsoft Paint software. Dark areas, double cut areas, and inappropriate areas were removed. Images were processed with Image J software (version 1.43; http://rsbweb.nih.gov/ij/download.html). Capillaries

were identified using a red filter with a sample window between 0-120, and counted with a pixel size of 8-150. Only pixel areas demonstrating a circularity factor between 0.3-1.0 were selected. The scale was adjusted and the area of the analysed section covered by muscle fibres was measured using a brightness filter with a standardized between 0-208. The number of capillaries per square millimetre was defined as capillary density and was averaged per subject. On average 302 ± 49 and 391 ± 60 capillaries were counted per muscle cross-section pre and post the skiing intervention, respectively. The cross sectional area of slow and fast type muscle fibres was previously assessed (Narici, Flueck 2011). In brief, frozen biopsies of *m. vastus lateralis* were sectioned at a separate occasion at twelve-micrometer thickness under a cutting plane that was adjusted in perpendicular direction to the major axis of the muscle fibres. The criteria for accepting a specific cutting angle were that the majority of the fibre profiles showed a ratio between the smallest and the largest diameter of 0.66 and above and that no further reduction of this ratio was apparent despite tilting the biopsy before sectioning. Cryosections were subjected to staining for type II myosin heavy chain and the signal was recorded digitally with an Axiocam MRc camera at a 10-fold magnification (Zeiss Axioskop 2 mot plus, Carl Zeiss Ltd, Welwyn Garden City, UK). We had previously shown that the majority of fibres were pure fibres. Therefore the cross-sectional area of stained (fast type) and unstained (slow type) fibres was determined against the scale by manually recording the fibre periphery of each assessed fibre within the ARDOM software (Degens et al. 2002). These numbers were used to calculate the percentage and MCSA of slow- and fast-type muscle fibres. On average, 56 slow- and 49 fast-type muscle fibres were counted per muscle cross-section.

Muscle biochemistry – Cryosections equivalent to a tissue volume of 10mm^3 were collected during sectioning and pooled frozen in a 2 ml Eppendorf tube. Total homogenate was prepared in cold RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate at 90% + fresh (1 mM NaF, 1 mM Na3VO4, 1 µg/ml leupeptin, 2 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM PMSF) with an Ultraturrax mixer on ice. Protein content was estimated against bovine serum albumine with the bicochinic acid kit (Pierce). SDS-extracts were prepared at 1 microgram/ microliter in SDS-Page loading buffer (50 mM Tris.HCL, pH 6.8, 10% glycerol, 2% SDS, 2%-mercaptoethanol, 0.1% bromphenol blue) by mixing and heating for 5 min at 95°C followed by a spin 1 min at 5000 g 25°C. The denatured proteins were then separated on a 7.5 or 10% SDS-PAGE on a Biorad mini electrophoresis system, western blotted on a nitrocellulose membrane subjected to immunodetection with commercial antibodies in a 0.05% Tween-Tris buffer system (TTBS) essentially as described (Flueck et al. 2011). The membrane was stained with Ponceau S before immunodetection to control for equal loading and protein transfer.

For mitochondrial proteins a 1:1000 dilution in TTBS/2.5% milk,/1% BSA of first anti-OxPhos Complex Kit from Invitrogen (catalog # 457999) composed of monoclonal antibodies against markers of complex I (NDUFA9), complex II (SDHA), complex III (UQCRC1), and complex V (ATP5A1) and 1:10000 dilution in TTBS/2.5% milk/1% BSA of secondary of horse radish peroxidase-conjugated goat anti-mouse antibody (ICN Biomedicals GMBH, Germany) was used. GLUT4 was detected with a 1:1000 dilution of polyclonal rabbit anti GLUT4 antiserum (MiIlipore, #07-1404) as two to three bands between 40 and 50 kDa as previously reported (Kristiansen &

Richter 2002) and 1: 5000 dilution of horse radish peroxidase-conjugated secondary anti-rabbit antibody from goat (ICN Biomedicals GMBH, Germany) in TTBS/2.5% milk/1% BSA , respectively. Signal detection was carried out with enhanced chemoluminescence (Femto kit, Pierce) and quantified with a Chemidoc system running under Quantity One software (Bio-Rad, Life Science Research, Hercules, CA, USA). The signal intensity of the respective band was estimated with the 'volume rectangular tool' and corrected for background of a band of equal height and size (area) in an empty sample lane. A paired loading design was employed to load equal amounts (20 microgram) of total homogenate against pre-stained marker Amersham RPN 756V for size determination. Background-corrected signals of proteins were normalised to actin and then normalized to the mean values of the respective samples before training to reveal relative protein concentrations.

Genotyping- The *ACE* genotype was assessed essentially as described in (Vaughan, Huber-Abel 2013) but with the modification that the reaction was run on homogenates of muscle cross sections in RIPA buffer.

Statistics – Statistical tests were calculated using Statistica 10.0 (Statsoft Inc, Tulsa, USA). Kolmogorow-Smirnow-Lilliefors-Test demonstrated that the assumption of a normal distribution could not be rejected at a p-value of 0.10. Post vs. pre skiing were assessed with a one-way repeated ANOVA with a two-tailed post-hoc test of Fisher. Linear relationships were assessed based on Pearson correlations at a p-value < 0.05.

Results

Downhill ski training-induced changes in body composition and exercise capacity: Tables 1 and 2 show the anthropometry of subjects, as well as exercise capacity and their changes with 3 months of downhill ski training. Body fat percentage was reduced by 8±2% but body weight was not affected (p=0.065; table 1). The thickness of *vastus lateralis* muscle was increased by 7±2% after skiing and this was matched by hypertrophy of slow type muscle fibres (table 1). Mass-specific VO_2max increased by 7±2% after the training program. Mass-specific VO_2max, body weight, fat content and the thickness of *vastus lateralis* muscle were not affected in the control subjects (tables 1 and 2).

Cardiovascular parameters with skiing: Heart rate at rest and after exertion demonstrated a trend for a reduction after ski training (p=0.06, table 3). Haematocrit was not significantly affected by ski training.

Adjustments of capillary processes with downhill ski training: Figure 1 shows a representative detection of capillary density in *vastus lateralis* muscle. Capillary density (+11 ± 5%) and capillary-to-fibre ratio (12 ± 5%) were increased after 3 months of skiing (Fig. 1).

Table 1: Anthropometry of subjects in function of skiing. Values represent mean + SEM. prior and post ski training. N=22, repeated ANOVA with post-hoc test of Fisher.

	pre	***post***	***p-value***
body weight [kg]	79.4 ± 2.9	78.1 ± 2.9	0.065
BMI [kg/m3]	27.1 ± 0.7	26.6 ± 0.7	0.062
body fat [%]	27.5 ± 2.4	26.8 ± 1.8	<0.001
vastus lateralis muscle			
thickness [mm]	189 ± 6	202 ± 7	0.001
CSA slow fibres [µm2]	5643 ± 412	6837 ± 568	0.040
CSA fast fibres [µm2]	4701 ± 490	5342 ± 454	0.070
Slow fibre area [%]	58 ± 5%	63 ± 4%	0.320
Capillary density [mm-2]	402 ± 35	431 ± 35	0.105

Table 2: Exercise capacity in function of skiing. Values represent mean + SEM prior and post ski training. N=22, repeated ANOVA with post-hoc test of Fisher.

	pre	***post***	***p-value***
Pmax [watts]	174.8 ± 45.1	176.7 ± 47.3	0.446
Watts/ kg	2.2 ± 0.5	2.3 ± 0.5	0.102
VO_2-max [ml/kg/min]	28.7 ± 5.0	30.7 ± 5.4	0.001

Table 3: Cardiovascular parameters in function of skiing. Values represent mean + SEM of heart rate (HR) in beats per minute (bpm) and blood pressure during exercise and haematocrit as estimated in entry and exit tests. N=22, repeated ANOVA with post-hoc test of Fisher.

heart rate	***pre***	***post***	***p-value***
HR rest (bpm)	71.6 ± 13.4	68.2 ± 9.3	0.065
HR max exertion (bpm)	153.3 ± 11.4	152.8 ± 13.2	0.062
HR 5 min recovery (bpm)	113.5 ± 15.2	111.0 ± 16.0	0.424
systolic blood pressure [mmHg]			
rest	125.2 ± 12.8	123.0 ± 13.3	
max exertion	207.6 ± 26.4	212.4 ± 23.0	0.150
5 min recovery (bpm)	160.5 ± 32.9	166.4 ± 32.4	0.461
diastolic blood pressure [mmHg]			
rest	79.5 ± 11.3	77.9 ± 9.6	
max exertion	88.8 ± 12.3	89.5 ± 13.3	0.839
5 min recovery (bpm)	72.6 ± 13.7	74.8 ± 16.5	0.635
Haematocrit	43.5 ± 2.6	42.6 ± 2.8	0.111

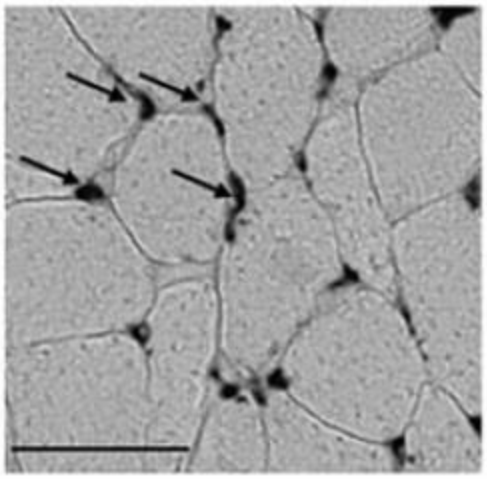
Table 4: Concentration changes of metabolic processes with ski training.
Mean ± SE of relative concentrations of selected metabolic factors in *m. vastus lateralis*. N=19, repeated ANOVA with post-hoc test of Fisher.

	pre	***post***	***p-value***
GLUT4 50-kDa	1.0 ± 0.2	1.3 ± 0.4	0.247
GLUT4 40-kDa	1.0 ± 0.2	1.1 ± 0.3	0.394
NDUFA9	1.0 ± 0.1	1.2 ± 0.1	0.139
SDHA	1.0 ± 0.1	1.1 ± 0.1	0.349
UQCRC1	1.0 ± 0.1	1.2 ± 0.1	0.101
ATP5A1	1.0 ± 0.1	1.2 ± 0.1	0.105

Table 5: ACE I/D polymorphism affect the response to ski training. Mean values of fold changes with skiing between the three genotypes resulting from the *ACE* I/D polymorphism and the corresponding p-value for the genotype-affected parameters (ANOVA).

fold change	***ACE-II***	***ACE-ID***	***ACE-DD***	***p-value***
VO_2-max [ml O2/min/kg]	1.01	1.06	1.13	0.042
Capillary length [mm]	1.09	1.36	1.85	0.049
Slow fibre area [%]	1.03	1.03	1.07	0.005
Heart rate at rest [bpm]	0.88	0.96	1.15	0.005

1a)



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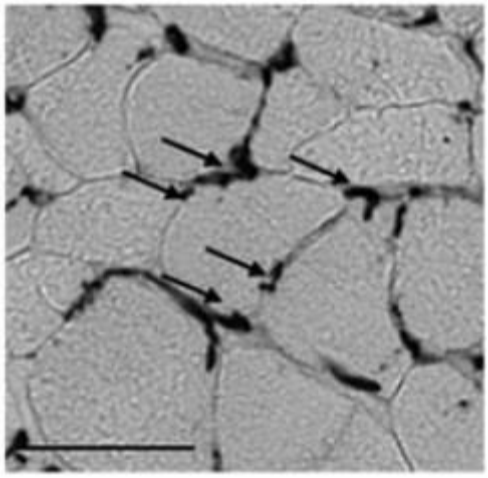

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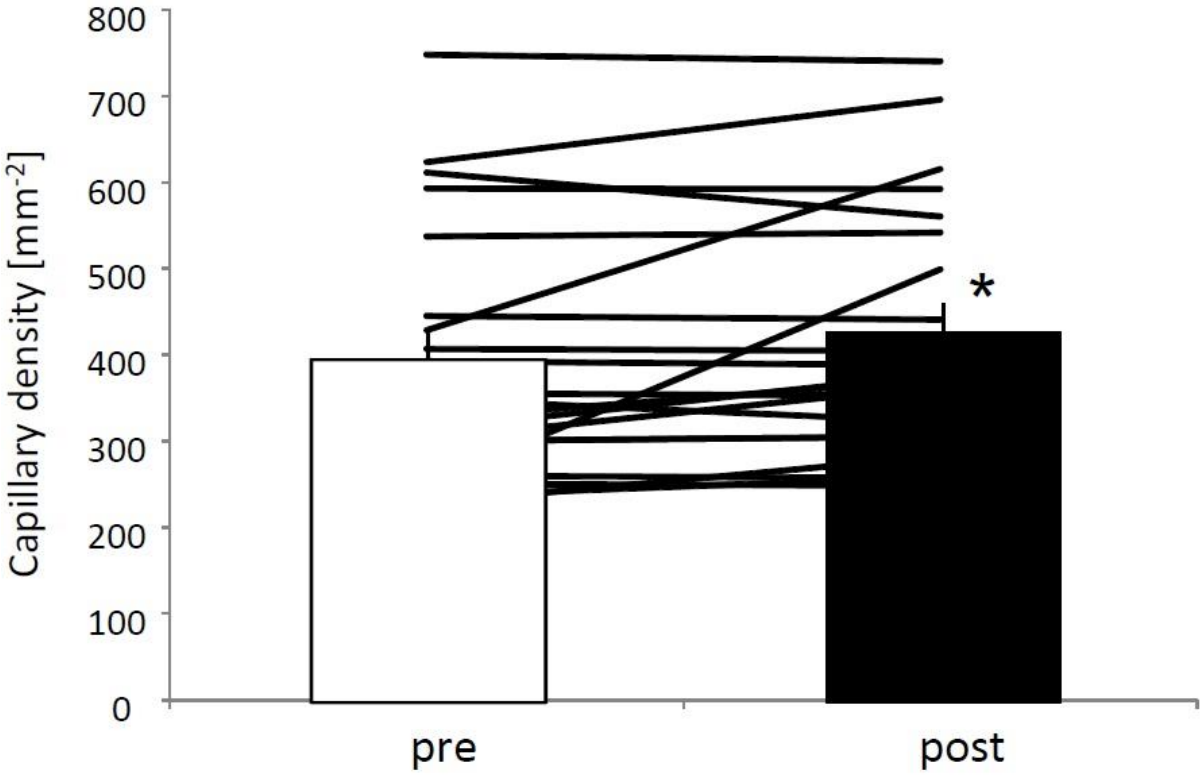

Figure 1a-d. Representative example of muscle capillarity. (a, top left; b top right) Example of detected capillaries in cross-sections of knee extensor muscle in a subject before (*a, top left*) and after (*b, top right*) ski training. Respective examples of stained capillaries are indicated with an arrow. Bar, 100 mm. (*c, bottom; d, page 161*) Bar graphs of mean ± SE and lines for individual changes in capillary density (c, bottom) and capillary-to-fiber ratio (*d, page 161*) before and after the 3 months of downhill ski training. n = 19. *P < 0.05 vs pre (repeated analysis of variance with post-hoc test of Fisher).

1d)

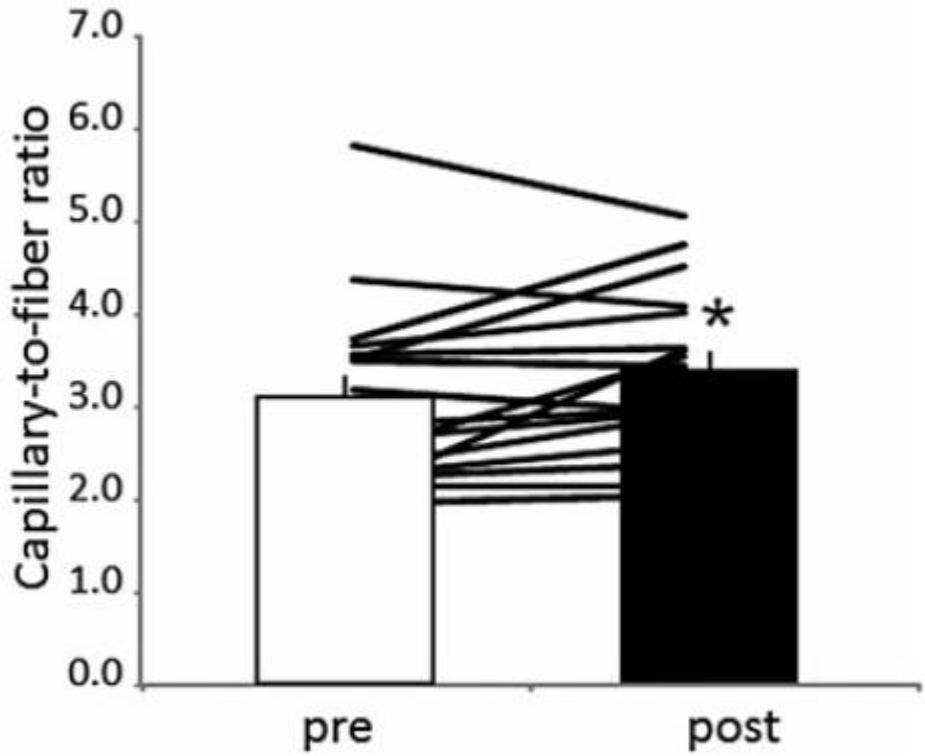

Figure1d: *for discription of figure 1 see page 160*

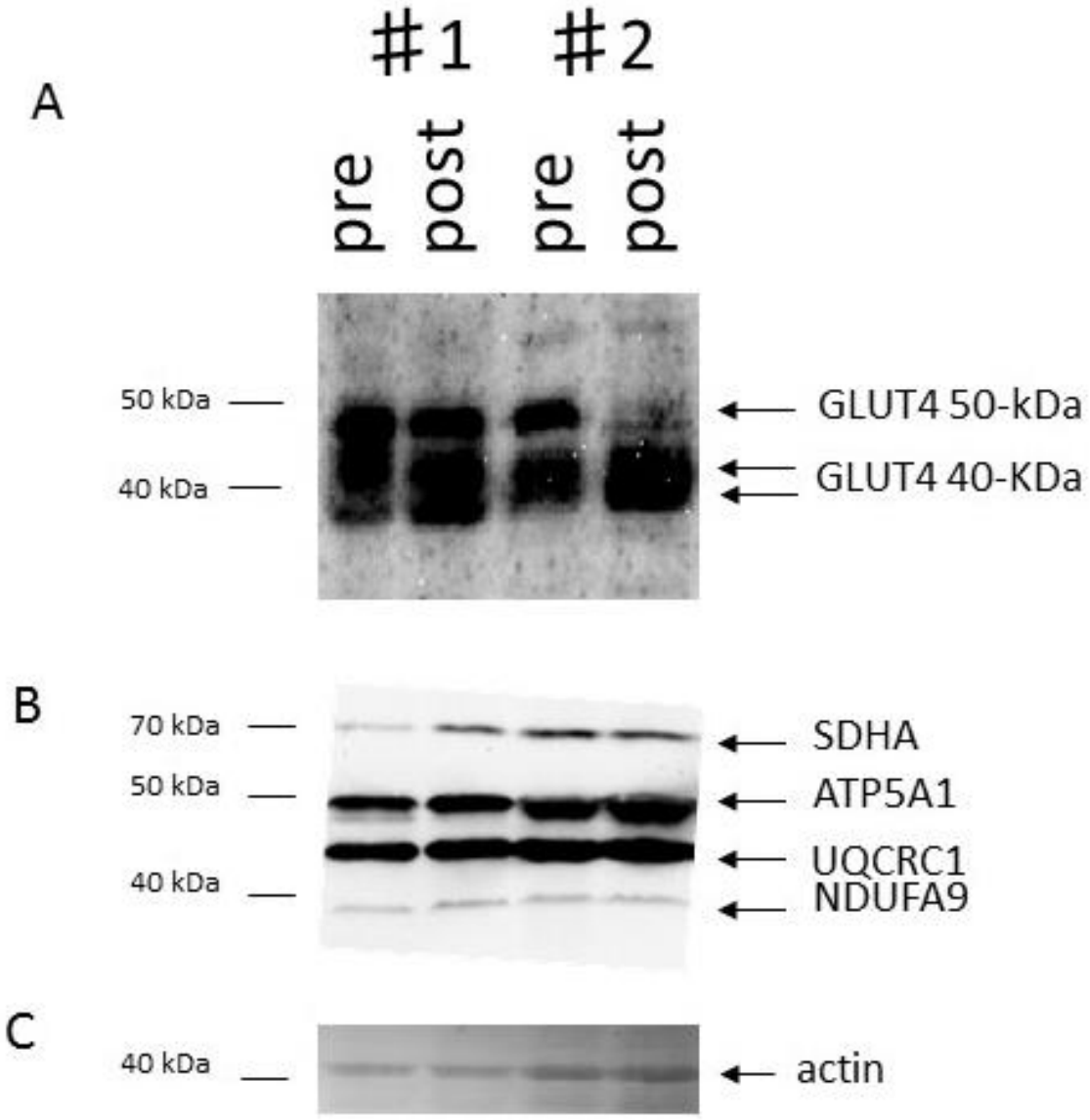

Figure 2: GLUT4 protein and respiratory chain constituent expression in human skeletal muscle with ski training. (a, b) Representative immunoblot of the detected GLUT4 immunoreactive protein at 40 and 50 kDa (a) and markers of four mitochondrial respiratory complexes I (NDUFA9), II (SDHA), III (UQCRC1), and V (ATP5A1) in vastus lateralis muscle in two subjects pre and post 3 months of ski training. (b) The respective bands and the weight of molecular markers are indicated. (c) Loading control of the Ponceau S stained membrane show skeletal alpha actin.

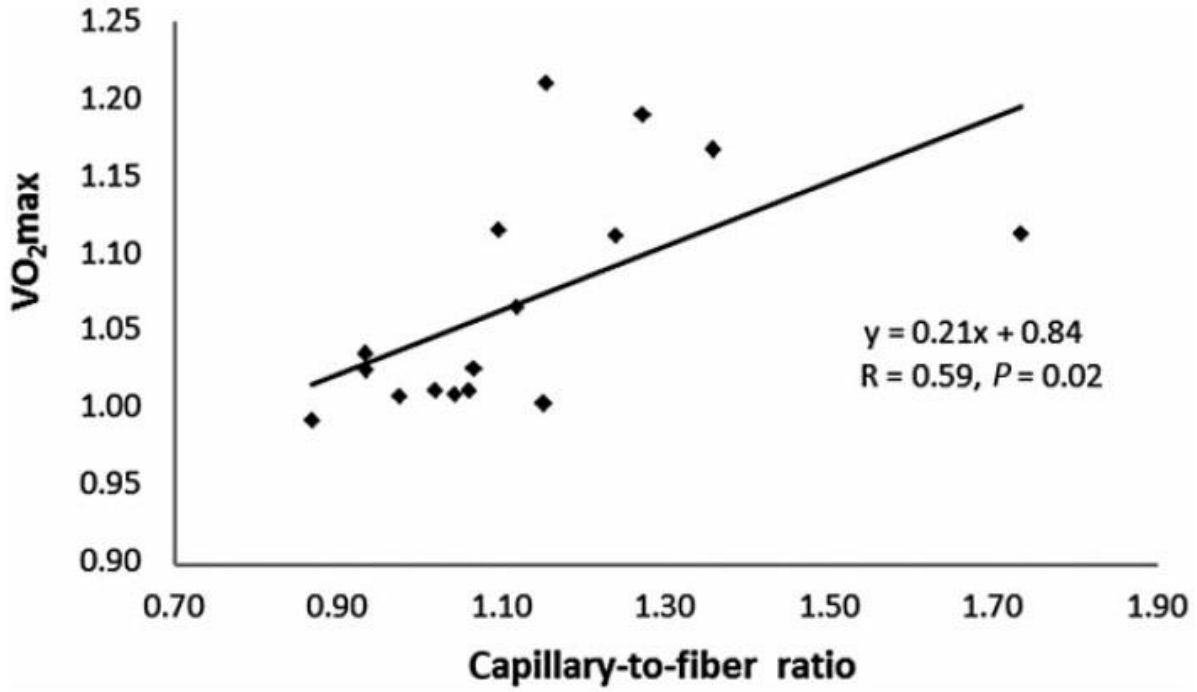

Figure 3: Correspondence between capillarity and maximal oxygen uptake ($VO_{2\text{-}max}$). Line graph showing the correlation between fold changes in capillary-to-fiber ratio in vastus lateralis muscle and $VO_{2\text{-}max}$ with 3 months of ski training.

Glucose transport with skiing: Three GLUT-4 protein bands, residing as a doublet near 40-kDa and a single band at 50-kDa (Hoshino et al. 2013; Kristiansen & Richter 2002), respectively were detected in *vastus lateralis* muscle (Fig. 2). Neither GLUT4 isoform was altered in relative concentration after ski training (table 4).
Unaffected expression of mitochondrial proteins with downhill skiing: Figure 1B shows representative immunoblots of the markers of the four mitochondrial respiratory complexes I (NDUFA9), II (SDHA), III (UQCRC1) and V (ATP5A1) in two subjects before and after the ski training. Concentrations of the mitochondrial markers were not significantly affected by ski training (table 4).

Correlations between local and system variables: There was considerable variability in the response of mitochondrial protein and capillarity to the skiing program. The fold-changes in capillary-to-fibre-ratio in *vastus lateralis* muscle correlated with the fold-changes in mass-specific VO_2max after ski training ($r=0.59$, $p=0.02$; Fig. 3). No correlations were observed between the fold-changes in mass-specific VO_2max and those of capillary density and the concentration of mitochondrial proteins after skiing. Before skiing, linear relationships for mass-specific VO_2max and the concentration of mitochondrial proteins were evident, i.e. SDHA ($r=0.56$), ATP5A1 ($r=0.53$). Variability in the response of VO_2max, heart rate at rest, and slow fibre area was related to an effect of the *ACE* I/D polymorphism (table 5).

Discussion

Downhill skiing is known to elevate glycogen metabolization during slow aerobic and forceful eccentric muscle actions downhill on the slope (Scheiber, Krautgasser 2009; Tesch, Larsson 1978; Tesch 1995) and can improve aerobic fitness in the elderly when performed repeatedly (Muller, Gimpl 2011). Maximal oxygen uptake during endurance exercise, as a measure of aerobic fitness, is set by the connected elements of the intra-organ path involved oxygen transport from the lungs to muscle mitochondria (di Prampero 1985). Thereby the larger portion (i.e. $3/4^{th}$) is allocated to cardiac output and oxygen binding to erythrocytes in blood with the remaining fraction being about equally partitioned between capillary cross section and mitochondria of working muscles. Here we show that the effect of ski training on mass-specific VO_2max is related to increases in capillary-to-fibre-ratio and capillary density but not mitochondrial protein concentration (compare Fig. 1 with table 4) in the major extensor muscle, *m. vastus lateralis*.

Increases mass-specific VO_2max after endurance training have been shown to be reflected by a locally exaggerated increase in mitochondria and capillary volume density (Hoppeler, Howald 1985). In line with the results in the former paper we identify a correlation between fold changes in mass-specific VO_2max and the changes in capillary-to-fibre-ratio with ski training (Fig. 3). The identified trends for a decrease in heart rate, both at rest and during exercise, further indicate that central adaptations occur with the repeated downhill skiing (table 3). These observations suggest that the effects of improved capillarity in peripheral muscle conspire with

central changes to explain gains in VO_2max with ski training of the elderly subjects of our study.

Downhill skiing involves in addition to shortening and isometric forms of contraction, also a considerable degree of lengthening type (i.e. eccentric) contractions (Berg & Eiken 1999). In this regard it is of interest to compare the in here identified adaptations in capillarity and thickness of knee extensor muscle to training protocols, which involve alike skiing involve eccentric type contractions and aerobic exercise (Scheiber et al. 2009). For instance, it has been pointed out that eccentric type of endurance exercise on a modified cycle ergometer produces 47% and 52% increases in capillary-to-fibre ratio and muscle cross sectional area, respectively, after 8-weeks of training while leaving mitochondrial density unchanged (LaStayo et al. 2000). These adaptations distinguish to the concomitant increase in mitochondrial volume density (+40%) and capillary-to-fibre ratio (+40%) with 6-weeks of the normal, 'concentric type', endurance training at 65% aerobic power on a cycle ergometer (Hoppeler et al. 1985). We have recently shown that adaptations in mitochondria with the latter endurance training protocol are reflected by increases of the concentration of mitochondrial proteins (NDUFA9, SDHA, *COX4I*1, UCP-3) in *m. vastus lateralis* by 60%-300% (Desplanches et al. 2013). While there is an appreciated influence of exercise intensity for training-induced alterations in mitochondrial enzymes in leg muscles (Schantz et al. 1983); the former observations emphasise that eccentric, as opposed to concentric type of endurance exercise, produce few adaptations in mitochondria in the recruited *vastus lateralis* muscle (Steiner et al. 2004). In contrast, the increased capillary-to-fibre ratio and capillary density, despite the expected dilution of capillaries in the larger *vastii*; highlight the

net increase in capillary supply in knee extensor muscle after ski training. Possibly this is explained by the role of mechanical factors as main stimulus for the growth of capillaries (Egginton 2011) as mechanical factors also are increasingly important with the eccentric type contractions during downhill turns on the slope (Berg & Eiken 1999).

An intriguing aspect of our study was that variability in the response of VO_2max and heart rate at rest was related to the I/D polymorphism in the gene for angiotensin converting enzyme, *ACE* (table 5). We have recently demonstrated that the *ACE* I/D polymorphism affects the change in mass-specific VO_2max and muscle capillarity of young subjects to endurance training on stationary cycle ergometers (Vaughan, Huber-Abel 2013). In that study subjects carrying the *ACE* I-allele were found to demonstrate larger increases in aerobic capacity than non-carriers of the I-allele which carry the D-allele only. On the other hand, in the study described here, subjects with the D-allele demonstrated larger improvements in VO_2max and capillary length in *m. vastus lateralis* after the moderate endurance stimulus. The present findings indicate a different influence of the *ACE* I/D polymorphism on the effects of concentric (i.e. cycle ergometer) versus eccentric (i.e. skiing) type of endurance exercise on aerobic capacity. Based on our data we hypothesize that different adjustments in heart rate, and possibly muscle perfusion, but the latter of which did not resolve at the level of statistical significance, contribute to the larger improvement in VO_2max in homozygous carriers of the *ACE* D-allele.

In contrast to our assumption, mitochondrial protein concentration was not increased in *vastus lateralis* muscle after the skiing program. These observations

indicate that adaptations of muscle energy supply lines with recreational skiing differ from the effects of recreational type of endurance training (Fluck & Hoppeler ; Suter et al. 1995). Our finding parallels the specific increase in capillarity (capillary-to-fibre ratio) with eccentric type, as opposed to concentric type, of endurance exercise (LaStayo, Pierotti 2000); suggesting that the observed adjustments in capillarity with skiing are driven by mechanical rather than metabolic factors.

Limitations: In our study a number of co-variables of oxygen transport were not assessed (i.e. stroke volume) and the levels of molecules assessed in our wet assay were expressed respective to relative signal intensities (per total protein or muscle cross section) and not respective to absolute SI units. Also, only one factor was assessed that is involved in glucose metabolism in skeletal muscle, which we hypothesised to be improved based on the increase in glucose metabolism with downhill skiing and the effects of endurance type training on the concentration of GLUT4, being involved in the import of blood-borne glucose (Desplanches et al. 2014; Scheiber et al. 2009; Tesch et al. 1978; Tesch 1995). However. GLUT4 is not a primary bottleneck in the regulation of glycolytic flux (Suarez et al. 2005) and glucose uptake and disposal was not measured. Therefore possible other modalities of GLUT4-related glucose handling cannot be excluded. Finally, we did not assess muscle parameters at the cellular and molecular level in the control group, which did not demonstrate a change in VO_2max compared to the skiing group (table 2). This is explained by ethical considerations related to the (minimally) invasive of the biopsy sampling technique. Although we cannot formally exclude a contribution of the repeated exposure to moderate altitude (i.e. 728-2700 m above sea level) to the

increased capillarity in *m. vastus lateralis* after ski training we do not identify correlations between vertical distance covered during the skiing intervention with VO_2max (r=-0.14, p=0.57), capillary density (r=-0.03, p=0.89), and capillary-to-fibre ratio (r=0.10, p=0.71).

Conclusion

Recreational downhill skiing in the generally healthy elderly produces an increase in capillarity rather than increases in respiratory chain constituents in a main muscle group operating with downhill action on the slope. The underlying mechanisms remain to be explored but appear to involve eccentric type contractions and genetic effects of the angiotensin converting enzyme.

Acknowledgements

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Author contributions

Contributions by Sander van Ginkel: performed *ACE* genotype measurements from biopsies (designed by Sv.G), cutting biopsies, staining, capillary counting, interpreted results of experiments, drafted, edited and revised the manuscript

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Chapter 6

Summarizing discussion

The aim of this research was to expose the pathway by which *ACE* and AngII are implicated in exercise-induced capillary growth of human muscle. In the introduction we hypothesize that the blunting of AngII production with concomitant exercise reduces improvements in metabolic fitness by removing an important stimulus for capillary growth in exercised muscle.

AngII, ACE genotype and exercise

In chapter 2 we described our investigation on the effect of exercise intensity and duration on serum AngII concentration. We observed that highest increases in indices of metabolic strain were observed after a maximal incremental exercise test, with a two-fold increase in serum AngII concentration after a very high intense 3-minutes exercise test. The relative change in AngII in this stucy compares well to the increase found in the study of Fagar et al. (1985). No significant increases in AngII were found after the 12-minutes test and maximal test with also no significant differences in AngII levels between the different time points after exercise (Fig. 2, page 46).

We did find that the increase in AngII after exercise is dependent on the *ACE* I/D gene polymorphism. After the maximal incremental exercise test subjects with the ACE-ID/DD genotype showed a decrease in serum AngII concentration after the maximal test while serum AngII concentration was not affected in subjects with the ACE-II genotype (Fig. 3; page 47) Thus, the *ACE* I/D genotype explains part of the

variation in serum AngII levels between subjects. This indicates that the rise in AngII post exercise seems to be exercise intensity and *ACE* genotype dependent.

It was further observed that subjects with the ACE-II genotype had a significant higher capillary perfusion in the finger after exercise compared to the ACE-ID/DD genotype. The observed trend for a lower capillary perfusion in the ACE-ID/DD genotype post exercise indicates that this body compartment is less perfused due to AngII-dependent vasoconstriction during exercise. We conclude that there seems to be a relationship between AngII levels and *ACE* genotype and between *ACE* genotype and blood flow.

Effects of ACE inhibition during exercise

We investigated the impact of *ACE* inhibition to expose the role of AngII in angiogenic gene expression in exercised muscle by assessing the effect of *ACE* inhibition by oral medication (chapter 3). The AngII values were 1.6 times higher after exercise in the *ACE* inhibition group and were not significantly increased in the control group. This increase in AngII of both groups is comparable with the increase found in the the study of Fagard (1985). In our study we further identified that *ACE* inhibitor intake increased production of pro-angiogenic factors associated with the capillaries in the interstitium, i.e. *VEGF* and *Tenascin-C* (Milkiewicz, 2001; Flück, 2000) after exercise and prevented the increase in oxygen dependent transcripts which are thought to mainly originate from muscle fibres (*COX4I2*, *HIF-1α*).

Influence of ACE on Tenascin C expression during exercise

We investigated whether Tenascin C expression in exercised muscle is controlled by *ACE* activity (chapter 4). We found an effect of *ACE* inhibition by the anti-hypertensive drug lisinopril on the response of *Tenascin-C* after exercise and identified that *Tenascin-C* transcript expression was only increased in an endurance control group and not in untrained control subjects. This indicates that elevated *Tenascin-C* expression post exercise relies on the inhibition of angiotensin modulated vasoconstriction. We provided with this study evidence for a role of the angiotensin-*Tenascin-C* axes during exercise-induced capillary growth and mitochondrial biogenesis in the muscle.

ACE genotype and exercise training

In the study described in chapter 5, we assessed with a training intervention whether the training response of the pathway that sets aerobic capacity would be modified by the *ACE* I/D gene polymorphism. 12 weeks of guided downhill skiing increased VO_2max, capillary density and capillary-to-fiber ratio, but not the concentration of metabolic proteins. Variability in the response of VO_2max and heart rate at rest was related to the *ACE* I/D genotype of the subjects. We concluded that the different adjustments in heart rate and possibly muscle perfusion, contribute to the larger improvement in VO_2max in subjects with the ACE-DD genotype. In this study we found that subjects carrying a D allele demonstrated larger improvements in VO_2max and capillary length after the training stimulus of recreational skiing.

The effect of ACE on AngII

As described in the introduction, serum AngII is produced by conversion of AngI via endothelium-related ACE. At rest subjects with the ACE-ID or ACE-DD

genotype demonstrate approximately 30% and 60% higher levels of *ACE* activity, respectively, compared to subjects of the ACE-II genotype (Rigat et al. 1990). Subjects with the ACE-DD genotype are as well presumed to have higher AngII generation and resting AngII levels (Danser et al. 2007). This assumption is confirmed with the data from our study where subjects with the ACE-ID had 6.0% higher AngII levels at rest compared with the ACE-II genotype, whereby the ACE-DD genotype had 2.8 times higher AngII levels (table 3, page 78). During exercise the renin-agiotensin system is stimulated and several studies show increases in serum AngII concentration after severe exercise, without taking the *ACE* genotype in consideration (Aldigier et al. 1993, Miura 1994, Staessen 1987, Braith 1992, Kato (1996), Danielsen 1988, Fagard 1985). We found that not all subjects showed an increase in AngII after exercise (chapter 2). Subjects with the ACE-II genotype had significant higher AngII values at all the time points after the maximal test then people without the I allele, who demonstrate decreased AngII values after the maximal test (page 46). On the identified effect of *ACE* genotype on the AngII levels after the maximal test, we think the *ACE* genotype exerts a major influence on the standard error. Subjects with the ACE-II/ID genotype had a higher increase in AngII after exercise than subjects with the ACE-DD genotype, who even showed a slight decrease in AngII levels post exercise. Apparently the natural *ACE* inhibition that subjects with the ACE-II/ID genotype do demonstrate at rest is overridden during exercise when the renin-angiotensin system is stimulated, resulting in an increase in renin activity and higher *ACE* levels. The fact that AngII is mainly increased after exercise in subjects having the ACE-II genotype could also explain why not all

studies find an increase in AngII after exercise (Shim et al. 2008, Blanchet et al. 2005).

When inhibiting *ACE* levels with an *ACE* inhibitor, we found that these subjects showed an increase in AngII after exercise, confirming that during exercise *ACE* activity is less inhibited than at rest without considering the role of shear stress in regulating *ACE* . Subjects who did not consume the medication also had an higher average serum concentration of AngII, but this did not reach the level of statistical significance (chapter 3). This lack of significance is probably related by the fact that 80% of the control subjects who did not take the *ACE* inhibitor appeared to have the ACE-DD genotype, and as stated before, subjects with this genotype have a less increase in AngII after exercise.

The effect of ACE inhibition

ACE inhibition is suggested to have a pro-angiogenic effect, with increasing capillary density and promotion of ischemia-induced angiogenesis (Gohlke et al. 1997; Fabre et al. 1999). During endurance exercise the mRNA expression of pro-angiogenic factors such as *VEGF* is increased in skeletal muscle (Gavin et al. 2000). In our study pro-angiogenic factors associated with the capillaries in the interstitium, i.e. *VEGF* and *Tenascin-C* were increased after exercise in the group who ingested the *ACE* inhibitor. This increase was much higher than in the group who did not take the *ACE* inhibitor. This highlights a possibl pro-angiogenic effect of *ACE* inhibitor in combination with endurance exercise.

Exercise in hypoxia produces a pronounced drop in muscle oxygenation. The oxygen dependent transcripts levels of *HIF-1α* and *COX4I2* are regulated in a hypoxia-modulated manner after cycling exercise (Desplanches et al. 2014). In our study *COX4I2* and *HIF-1α* were decreased after exercise in the group who took an *ACE* inhibitor (chapter 3, figure 2). Our findings support the view that an angiotensin-regulated mechanism affects the hypoxia-specific gene response in peripheral muscle to endurance exercise and support the notion that the muscle's transcript regulation by *ACE* inhibition is related to muscle oxygenation during exercise. This indicates that there is a shift in the activation of the gene program from muscle fibres to the surrounding interstitium after *ACE* inhibition.

The effect of training upon ACE

Endurance training increases the aerobic capacity. Subjects with the ACE-II/ID genotype (natural inhibition of ACE) are used to have larger increases in aerobic capacity than subjects with the ACE-DD genotype after bicycle exercise training (Vaughan et al. 2013, Defoor et al. 2006). Increases in VO_2-max after endurance training have been shown to be reflected by a locally exaggerated increase in mitochondria and capillary volume density (Hoppeler et al. 1985). In line with those results we identify a correlation between fold changes in VO_2-max and the changes in capillary-to-fibre-ratio with ski training (Fig. 3, page 163). The identified trends for a decrease in heart rate, both at rest and during exercise, further indicate that central adaptations occur with the repeated downhill skiing (table 3, page 157). These observations suggest that the effects of improved capillarity in peripheral

muscle conspire with central changes to the gain in VO_2max with ski training for the elderly subjects of our study.

An intriguing aspect of our study was that variability in the cardiovascular response, based on measures of VO_2-max and heart rate at rest, was related to the *ACE* I/D genotype (table 5, page 159). *ACE* I/D polymorphism affects the change in VO_2max and muscle capillarity of young subjects to endurance training on stationary cycle ergometers (Vaughan et al. 2013). In that study subjects carrying the *ACE* I-allele were found to demonstrate larger increases in aerobic capacity than non-carriers of the I-allele, which only carry the D-allele (i.e. ACE-DD genotypes). On the other hand, in our study, subjects with the D-allele demonstrated larger improvements in VO_2max and capillarity in m. vastus lateralis after a moderate endurance exercise stimulus. The present findings indicate a different influence of the *ACE* I/D polymorphism on the impact of concentric (i.e. cycle ergometer) versus eccentric (i.e. skiing) type of endurance exercise on aerobic capacity. Based on our data we hypothesize that different adjustments in cardiac performance, and possibly capillary perfusion of skeletal muscle, although the latter did not resolve at the level of statistical significance ($p=0.17$), contribute to the larger improvement in VO_2-max in homozygous carriers of the *ACE* D-allele.

Factors affecting ACE levels

We have shown that the different levels of *ACE* can influence the effects of training. In our studies we saw that the *ACE* genotype is an important factor that plays a role. As differences in *ACE* levels between subjects can be accounted for about 20% for the I/D polymorphism (Danser et al. 2007), several additional factors must play a

role. As we have tried to control external factors that may influence the levels of circulating ACE, we may cannot generalize our conclusions to the whole population. Important factors that influence the *ACE* levels are gender, race, age, medical condition and BMI (provide a reference). We have studied healthy Caucasian males between 20 and 45 years of age, non-diabetic, non-hypertensive with a BMI between 20 and 30 kg/m^2. As *ACE* and AngII levels have a circadian rhythm we tested within each subjects at the same time of the day when coming multiple times for a test. As we tested multiple subjects on one day we tested not all participants at the same time of the day. The circadian rhythm is also very hard to control for as subjects have different circadian rhythm of *ACE* and AngII.

Limitations and further studies

A limitation of this research is that it was not built on a cross-over design. This was explained by ethical and temporal constraints imposed for such an investigation under the granted doctoral fellowship. For the same reason a minimal number of subjects, was recruited in our investigation. This number was based on a prospective power analysis of pilot data with hypertensive subjects to meet the criteria for achieving a statistically significant difference for Tenascin-C transcript level changes pre vs. post endurance training (Zoll et al. 2006, unpublished).

In order to identify a possible interaction effect between the ACE I/D gene polymorphism and Lisinopril an increase of the number of subjects would have been required to an extent that was out of the scope of the current investigation. As we have shown, the *ACE* I/D gene polymorphism influences the muscle response to

exercise, suggesting a critical role of ACE-modulated vascular tone, which is important for the regulation of exercise response. A study investigating in detail what the effect is of the *ACE* genotype in relation to exercise would be required. Such a study should contain sufficient subjects with the ACE-II, ACE-ID and ACE-DD genotype to be able to compare the different genotypes. In my latest paper, that was published during the revision of this thesis, we found that besides a regulation of the ACE protein, Lisinopril exerts effect on oxygen-modulated gene transcripts (*COX4I1*, *COX4I2* and *HIF-1a*) and the *ACE* transcript itself in skeletal muscle. (Mathes et al. 2015). In this study we concluded that the response of ACE and COX4I2 transcripts post-exercise depends on the ACEI/D genotype. Despite the pronounced effects that we found in this study, caution applies when extending conclusions from our data to the general population because possibly many confounding variables interfere, which in our investigation were controlled by having subjects undergo a dominant one-legged exercise stimulus under standardized conditions. At the moment M. Flück is carrying out active research upon this topic.

Population

The population we measured in our study consisted of Caucasian men in the age group between 20 and 55 years of age. Most of the people who take *ACE* inhibitors are generally elderly. Within a subject, AngII increases above 55 years of age (Tsunoda et al. 1986). In the study of Chiang et al. (2006) it was shownin a large cohort study that elderly people with the *ACE* D-allele have a higher risk on developing hypertension. So when becoming older, the effect of ACE inhibition will be higher. The effect of ACE inhibition in our study, if we had tested older subjects,

would probably have been more pronounced. In this regard we think that the effect of the *ACE* genotype upon training and exercise is even stronger in the elderly.

Effect on Health Care practice

We have shown that there are differences in AngII production after exercise between subjects having different *ACE* levels. AngII-targeted therapies are the current default treatment for hypertensive patients. Our findings that physiological improvements after exercise are dependent on *ACE* levels, both due to different *ACE* genotypes and to taking *ACE* inhibitors could have clinical repercussions. Differences in exercise-induced improvements have rarely been valued in pharmacological studies of hypertension and exercise rehabilitation (Böhm et al. 2008). Awareness of the existence of such an influence of *ACE* levels could develop a major impact on current Health Care practice, particularly in exercise rehabilitation when one wants to exploit the sympatholytic and angiogenic benefits of physical therapy (Saltin et al.). It may explain the individual variation in the response to exercise rehabilitation in aerobic power and oxygen uptake (VanHees et al. 2005, Defoor et al. 2006). As of now the dose of the prescribed *ACE* inhibitors is only based on the severity of the hypertension, but this could be significantly improved by taking the *ACE* genotype of the patient into account. As we have shown, establishing the *ACE* genotype of a person can be easily obtained with a protocol using an ear bud twirling against the inner cheek. People with lower AngII levels at rest (e.g. ACEII genotype) could be prescribed a lower dose of *ACE* inhibitors. Besides establishing the *ACE* genotype it is crucial to know the physical activity level of the person. It is known that excercise has a positive influence upon the blood pressure. We have shown that the *ACE*

inhibition has influence on the physiological improvements in subjects who are already physical active. We assume that the found effects would be even stronger in inactive subjects. When prescribing ACE inhibitors it would be recommendend to take besides of the severity of the high blood pressure also the *ACE* genotype of the person and its activity level into account into account. The same profits, with even higher physiological improvements, could be obtained with a lower doses in many patients. As *ACE* inhibitors are widely prescribed, it could save costs for health care as well.

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