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Plasma asymmetric dimethylarginine concentrations are not related to differences in maximal oxygen uptake in endurance trained and untrained men

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NEW FINDINGS

What is the central question of this study?

Asymmetric dimethylarginine (ADMA) is related to exercise capacity in patients with cardiovascular diseases. However, no studies have investigated whether there are associations of plasma ADMA concentrations with oxygen (O₂) delivery and subsequently exercise capacity in healthy subjects without potentially confounding influence of inflammation and oxidative stress.

What is the main finding and its importance?

Plasma ADMA concentrations are not related to exercise capacity in healthy subjects, while O₂ delivery in the working skeletal muscle during maximal graded-exercise test is not associated with any of L-arginine analogs. These findings demonstrate that ADMA alone does not play a crucial role in local muscle perfusion and in maintaining exercise capacity.

ABSTRACT

Purpose: Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthesis that could limit oxygen (O₂) delivery in the working skeletal muscles by altering endothelium-dependent vasodilation. Exercise capacity is associated with plasma ADMA concentrations in patients with cardiovascular diseases, but this issue has still not been investigated in healthy subjects. We aimed to determine whether plasma ADMA concentrations were negatively associated with exercise capacity in young healthy male subjects.

Methods: Ten men with maximal oxygen uptake ($\dot{V}O_{2\max}$) > 65 mL·kg⁻¹·min⁻¹ were included in the high exercise capacity group (HI-FIT), and ten men with $\dot{V}O_{2\max}$ < 45 mL·kg⁻¹·min⁻¹ were included in the low exercise capacity group (LO-FIT). Plasma ADMA and other L-arginine analogs concentrations were measured before and after a maximal graded-exercise test by liquid chromatography–tandem mass spectrometry. Microvascular O₂ delivery during exercise was estimated through the pattern from the sigmoid model of muscle deoxygenation in the vastus lateralis measured by near infrared spectroscopy (NIRS).

Results: $\dot{V}O_{2\max}$ was 60% higher in the HI-FIT group (median: 70.2; IQR: 68.0–71.9) than in the LO-FIT group (median: 43.8; IQR: 34.8–45.3). Plasma ADMA concentrations did not differ between the LO-FIT and HI-FIT groups before (0.50 ± 0.06 vs. 0.54 ± 0.07 μmol·L⁻¹, respectively) and after maximal incremental exercise test (0.49 ± 0.08 vs. 0.55 ± 0.03 μmol·L⁻¹, respectively). There was no significant association of plasma ADMA concentrations with the pattern of local muscle deoxygenation and exercise capacity.

Conclusion: Exercise capacity and microvascular O₂ delivery are not related to plasma ADMA concentrations in young healthy male subjects. Our findings show that ADMA does not play a crucial role in local muscle perfusion and in maintaining exercise capacity without pathological conditions.

Abbreviations: *A*: amplitude of [HHb] response; ADMA: asymmetric dimethylarginine; *c*: constant dependent of *d*, *c/d*: x-value corresponding to 50 % of [HHb] total amplitude; *d*: slope of [HHb] sigmoid model; *f*₀: baseline % Δ [HHb]_{max}; Hb: hemoglobin; [HHb]: vastus lateralis muscle deoxygenation; Δ [HHb]: relative changes in [HHb] from baseline values; HI-FIT: subjects with high exercise capacity; LC–MS/MS: liquid chromatography–tandem mass spectrometry; LO-FIT: subjects with low exercise capacity; Mb: myoglobin; NO: nitric oxide; NIRS: near infrared spectroscopy; RER: respiratory exchange ratio; SDMA: symmetric dimethylarginine; $\dot{V}E$: minute ventilation; $\dot{V}CO_2$: CO₂ output; $\dot{V}O_2$: oxygen uptake; $\dot{V}O_{2max}$: maximal oxygen uptake; WR: work rate. **INTRODUCTION**

Peak exercise capacity is a well-established independent factor determining cardiovascular health and predicting cardiovascular mortality (Williams, 2001; Myers *et al.*, 2002), and is most often assessed by measurement of maximal oxygen uptake ($\dot{V}O_{2max}$) during incremental exercise test until exhaustion. $\dot{V}O_{2max}$ is mostly determined by the ability to increase cardiac output and blood flow to the working skeletal muscle to meet the increased vasodilator activity during exercise (Bassett & Howley, 2000). Therefore, a blunted increase in working muscle blood flow due to microvascular dysfunction may alter muscle O₂ delivery and exercise capacity in pathological conditions (Takagi *et al.*, 2014). Nitric oxide (NO) is the main factor involved in the relaxation of vascular smooth muscle cells, and is enzymatically synthesized by NO synthase from L-arginine in endothelium (endothelial NOS, eNOS) (Cooke, 2004) and muscle (neuronal, nNOS) cells (Nakane *et al.*, 1993). During exercise, NO production is stimulated by shear stress in vascular walls (Cooke, 2004) and increased Ca²⁺ levels in muscle cells (Nakane *et al.*, 1993). It has been shown that exercise capacity was positively associated with NO-dependent endothelium function in patients with cardiovascular diseases (Hambrecht *et al.*, 1998) and with the formation of NO in healthy humans (Jungersten *et al.*, 1997). Furthermore, 3-weeks of omega-3 fatty acids supplementation increase serum NO concentrations and $\dot{V}O_{2max}$ in endurance-trained athletes (Żebrowska *et al.*, 2015). After omega-3 fatty acids supplementation, changes in $\dot{V}O_{2max}$ are associated with increased NO formation and improved endothelial function in conduit brachial artery (Żebrowska *et al.*, 2015). These findings suggest that NO bioavailability can be a factor that contributes to O₂ delivery in the working skeletal muscles and sustain $\dot{V}O_{2max}$ even in endurance-trained subjects.

Impaired exercise capacity observed in patients with cardiovascular diseases such as chronic heart failure or peripheral arterial disease is associated with increased plasma asymmetric dimethylarginine (ADMA) concentrations and decreased L-arginine to ADMA ratios (Wilson *et al.*, 2010; Seljeflot *et al.*, 2011). ADMA is an endogenous competitive nitric oxide (NO) synthase inhibitor that is produced by methylation of L-arginine residues of intracellular proteins (Blackwell, 2010). An increase in plasma ADMA concentrations, under pathological conditions, can increase oxidative stress and inhibit NO-mediated vascular smooth muscle cells relaxation (Vallance *et al.*, 1992). The impaired NO signaling by ADMA is associated with reduced flow-mediated dilatation and increased vascular resistance even in subjects with low cardiovascular risk (Kielstein *et al.*, 2004; Ardigo *et al.*, 2007). Lessened vasodilation related to ADMA could impair the ability to increase muscle blood flow and O₂ delivery in response to the increased O₂ demand of working muscle (Sperandio *et al.*, 2012), and hence contribute to the low rate of maximal oxygen uptake ($\dot{V}O_{2max}$) that characterizes low exercise capacity in pathological conditions (Takagi *et al.*, 2014). Although a negative association between exercise capacity and ADMA has been showed in patients with cardiovascular diseases, it is nevertheless unknown whether $\dot{V}O_{2max}$ and muscle O₂ delivery are also negatively associated with

ADMA in the absence of pathological conditions or aging. As such, ADMA could play a crucial role in the regulation of blood flow and O₂ delivery in the working skeletal muscle, and might be related to differences in exercise capacity without inflammation process and oxidative stress.

The primary purpose of the present study was therefore to determine whether plasma ADMA concentrations were negatively associated to exercise capacity in young subjects with no history of cardiovascular or metabolic diseases. We hypothesized that ADMA concentrations would be lower in highly trained endurance athletes characterized by high $\dot{V}O_{2\max}$ than in untrained but healthy subjects with low $\dot{V}O_{2\max}$. A secondary purpose was to determine whether differences in the pattern from the sigmoid model of muscle deoxygenation, assessed by Near Infrared Spectroscopy (NIRS), during exercise were associated with plasma concentrations of ADMA and other L-arginine analogs. As suggested by Ferreira *et al.* (2007), we hypothesized that plasma ADMA concentrations would be positively associated with the shift of muscle deoxygenation pattern to the left that reflects diminished microvascular function.

Material and Methods

Ethical approval

The present study was approved by the Institutional Ethics Committee of Provincial School of Hainaut (HEPH)-Condorcet (reference no. HEPHC-2016-012) and complied with the guidelines set out in the Declaration of Helsinki, except for registration in a database. The study protocol, purpose and risks were described to each study participant before obtaining written consent.

Population

Twenty young men free from any cardiovascular, respiratory, renal or metabolic disease by history or clinical examination were included in the study. Ten subjects contacted within local running and triathlon clubs were included in the group with high exercise capacity (HI-FIT). Ten subjects drawn from a list of volunteers for exercise studies and who did not engage in more than 3 hours of structured training per week were included in the group with low exercise capacity (LO-FIT, n=10). The criteria for inclusion in the HI-FIT group were $\dot{V}O_{2\max} > 65 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and/or aerobic peak power within $4.9\text{-}6.4 \text{ W}\cdot\text{kg}^{-1}$; the criteria for inclusion in the LO-FIT group were $\dot{V}O_{2\max} < 45 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and/or aerobic peak power $< 4.0 \text{ W}\cdot\text{kg}^{-1}$. The peak power output criteria were based on the Guidelines to Classify Subject Groups in Sport Science Research and corresponded respectively to performance level 4 for the HI-FIT group and performance level 1 for the LO-FIT group (De Pauw *et al.*, 2013).

The sample size was determined using the results from the study by Tanahashi *et al.* (Tanahashi *et al.*, 2014b) which compared plasma ADMA concentrations in two groups of postmenopausal women that differed significantly for $\dot{V}O_2$ at the ventilatory threshold (VO_2VT). Plasma ADMA concentration was $0.58 \pm 0.14 \mu\text{mol}\cdot\text{L}^{-1}$ in women with low VO_2VT ($10.7 \pm 0.7 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and $0.65 \pm 0.08 \mu\text{mol}\cdot\text{L}^{-1}$ in women with high VO_2VT ($14.3 \pm 1.6 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$).

As this study was conducted with two groups of subjects whose $\dot{V}O_{2\max}$ differed by at least 20 mL·kg⁻¹·min⁻¹ (see inclusion criteria) we assumed a difference of 0.15 μmol·L⁻¹ in plasma ADMA concentrations between the two groups and an estimated pooled standard deviation of 0.11 μmol·L⁻¹; the sample size was calculated to be n=10 in each group to achieve a statistical power of 80% and a significance level of 5% for bilateral comparison. Power analysis was performed using SAS University Edition (SAS Institute Inc., Cary, NC, USA).

The characteristics of the two groups are presented in Table 1. Subjects were not taking any medication known to affect NO metabolism, cardiorespiratory and haemodynamic responses to exercise.

- Table 1 here -

Experimental protocol

Subjects attended to the laboratory to complete a graded cycling exercise test conducted until volitional exhaustion to determine $\dot{V}O_{2\max}$ and the profile of muscle deoxygenation. Oxygen uptake and other gas exchange parameters were measured by indirect calorimetry. Local changes in deoxyhemoglobin ([HHb]) in the vastus lateralis muscle were measured by Near Infrared Spectroscopy (NIRS). Venous blood samples were drawn before and after exercise to determine plasma ADMA and L-arginine analogs concentrations.

Maximal graded exercise test

$\dot{V}O_{2\max}$ and aerobic peak power were measured during a graded cycling exercise test conducted until volitional exhaustion performed on a mechanically braked cycle ergometer (894E, MONARK EXERCISE, AB, Sweden). Baseline work rate (WR) and WR increments were adapted to each of the two groups to achieve exhaustion within 12 to 20 minutes after the start of the test. Baseline WR was 100 W and 35 W in the HI-FIT and LO-FIT groups respectively, and WR increments were 35 W and 20 W every 2 min in the HI-FIT and LO-FIT groups respectively. We considered that $\dot{V}O_{2\max}$ was achieved when at least three of the following criteria were met: volitional exhaustion; theoretical maximal heart rate ($220 - \text{age} \pm 10$ beats·min⁻¹), Respiratory Exchange Ratio (RER: $\dot{V}CO_2/\dot{V}O_2$) above 1.1; and a $\dot{V}O_2$ plateau (2.1 mL·kg⁻¹·min⁻¹) between the last two stages. Aerobic peak power was calculated as the sum of power output during the last stage fully completed and the WR increment multiplied by the fraction of time spent in the final unfinished stage.

Gas exchange measurement

$\dot{V}O_2$, CO₂ output ($\dot{V}CO_2$), RER, Minute Ventilation ($\dot{V}E$) were measured breath-by-breath with a gas exchange measurement system (K4b₂, COSMED, Italy). The gas analyzer was calibrated before each test with ambient air and a gas mixture of known composition (O₂: 16 %, CO₂: 5 %). The flowmeter was calibrated with a 3-liter syringe. Gas exchange data were recorded on a computer for later analysis. Heart rate was continuously recorded with a heart rate monitor (S810, POLAR, Finland).

Muscle deoxygenation

Vastus lateralis muscle deoxygenation ([HHb]) was continuously measured during the exercise test by NIRS (Oxymon, ARTINIS MEDICAL SYSTEM, Netherlands). Briefly, the NIRS system consisted of an emission probe (780 and 850 nm) that carries the NIRS light produced by laser diodes to the tissue of interest and a detector probe that transmitted light returning from tissue to spectrometer. In muscle tissue, NIRS signals result from changes in light absorption by hemoglobin (Hb) and myoglobin (Mb), which is related to their O₂-dependent status. The emission and detector probes were spaced out of 4 cm and were positioned longitudinally on the belly of the muscle vastus lateralis at midway between the lateral condyle of the knee and the greater trochanter of the femur. Probes were taped to the skin with adhesive strapping and covered with a dense black cloth to minimize exogenous light contamination, and then wrapped with an elastic bandage to avoid any movement during maximal exercise test. NIRS signal acquisition was performed at 10 Hz and data were finally exported at 1 Hz for later analysis.

Muscle deoxygenation and $\dot{V}O_2$ data analysis

Analysis of muscle deoxygenation patterns was based on the parameters of the sigmoid model fitted to the change of [HHb] according to the principles reported by Boone *et al.* (2010) during incremental step exercise.

Regarding $\dot{V}O_2$ data, aberrant data points that went outside 4SD filter-band of local mean were removed, and data were subsequently interpolated to 1 s intervals. $\dot{V}O_2$ data were left-shifted by 20 s to account for the circulatory time lag between muscles and lungs to match changes in $\dot{V}O_2$ related to muscles with pulmonary $\dot{V}O_2$ (Murias *et al.*, 2011). Due to the uncertainty of optical path length in the muscle vastus lateralis, exercise [HHb] data were expressed as relative changes (Δ [HHb]) from baseline values, which corresponded to the end 30-s of resting 2-min recording of [HHb] in seated position. $\dot{V}O_2$ and Δ [HHb] responses were time-aligned and normalized such as 100 % represented the average of the final 30 s of the last step that had to be sustained at least 90 s by the subject. Given the interindividual differences in time-to-exhaustion during maximal exercise test, $\dot{V}O_2$ and HHb responses were reduced into 100 equal bins to allow a direct comparison between the HI-FIT and LO-FIT groups.

The responses of normalized Δ [HHb] as function of % $\dot{V}O_{2\max}$ during the maximal graded exercise test were fitted by a sigmoid model as follows:

$$F(x) = f_0 + A/(1 + \exp^{-(-c+dx)})$$

where f_0 represents the baseline % Δ [HHb]_{max}, A is the amplitude of the response, d is the slope of the sigmoid model, c is a constant that is dependent of d , c/d is the x-value corresponding to 50 % of the response total amplitude.

Figure 1 displays the muscle deoxygenation profiles of two subjects representative of the HI-FIT and LO-FIT groups.

- Figure 1 here -

Blood samples

Blood samples were drawn into EDTA tubes from an antecubital vein before and 15 minutes after the exercise test with subjects in a semi recumbent position. Blood samples were centrifuged at 3600 g and 4 °C for 10 min, and plasma was aliquoted and stored at -80°C for later analysis. Plasma concentrations of L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and homoarginine were measured by a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method (Shin *et al.*, 2011; Martens-Lobenhoffer *et al.*, 2013). LC–MS/MS methods are considered as the “gold standard” for the measurement of these analytes (Martens-Lobenhoffer & Bode-Böger, 2007).

Statistical analysis

The complete analysis of data and the fitting of sigmoid models were run using the software R (R version 3.2.5, R Foundation for Statistical Computing, Vienna, Austria). Data were reported as mean \pm standard deviation (SD) unless otherwise stated. Normal distribution and heteroscedasticity of the data were assessed by the Shapiro-Wilk test and the Levene test, respectively. Characteristics of subjects were compared with the Student’s t-test for independent samples or the Mann-Whitney *U* test when parametric conditions were not satisfied. The goodness-to-fit of sigmoid models by R^2 value, the residual sum of squares, and the standard error estimate were compared between the LO-FIT and HI-FIT groups with the Mann-Whitney *U* tests. Parameters derived of the sigmoid model of $\Delta[\text{HHb}]$ responses as function of $\% \dot{V}O_{2\text{max}}$ during maximal exercise test were also compared with the Mann-Whitney *U* test. Comparisons regarding absolute plasma L-arginine analogs concentrations and ratios were analyzed with two-way ANOVA to assess the effects of exercise capacity (HI-FIT vs LO-FIT) and time (pre- vs. post-exercise), and potential interaction of exercise capacity and time. Post-hoc analysis was then performed with Benjamini-Hochberg correction for independent and dependent samples, as appropriate. The associations between the plasma concentrations of L-arginine analogs and $\dot{V}O_{2\text{max}}$ were tested with Pearson correlation coefficients. Associations of $\Delta[\text{HHb}]$ sigmoid responses during maximal exercise test with plasma L-arginine analogs concentrations and exercise capacity were examined with Spearman's rho. Statistical significance was set for all tests at $p < 0.05$.

RESULTS

Plasma ADMA concentrations

Results are displayed in Figure 2a. There was no significant group or time effect on plasma ADMA concentrations, although there was a trend for higher concentrations in the HI-FIT group ($p = 0.06$). There was no significant association between pre- or post-exercise plasma ADMA concentrations and $\dot{V}O_{2\text{max}}$. There was also no significant association between plasma ADMA concentrations at any time and parameters derived from the sigmoid model of muscle deoxygenation assessed by NIRS.

– Figure 2 here –

Plasma L-Arginine analogs concentrations and ratios

The plasma concentrations of L-arginine were significantly lower after exercise compared to baseline ($p < 0.05$, Figure 2b).

After exercise, the L-arginine to ADMA ratio was significantly higher in the LO-FIT group than in the HI-FIT group ($p < 0.05$, Figure 2c). The homoarginine to ADMA ratio was significantly higher in the LO-FIT group than in the HI-FIT group after exercise ($p < 0.05$), and increased significantly from before to after exercise in the LO-FIT group only ($p < 0.05$, Figure 2d).

The L-arginine to ADMA ratio was significantly associated to $\dot{V}O_{2\max}$ before and after exercise ($r = -0.47$, $p < 0.05$, $r = -0.58$, $p < 0.01$, respectively). The homoarginine to ADMA ratio was negatively associated to $\dot{V}O_{2\max}$ after, but not before, exercise ($r = -0.56$, $p < 0.01$). $\dot{V}O_{2\max}$ was significantly associated with the plasma concentrations of homoarginine after exercise ($r = -0.48$, $p < 0.05$). Plasma concentrations of SDMA and homoarginine are displayed in Figure 3a and b respectively. All correlations between plasma L-arginine analogs concentrations and $\dot{V}O_{2\max}$ are reported in Table 2.

- Figure 3 here -

- Table 2 here –

Muscle deoxygenation patterns during exercise

The slope of the sigmoid response (d) and the c/d point corresponding to 50 % of the total amplitude did not significantly differ between the LO-FIT and HI-FIT groups. There was no association between the plasma concentrations of L-arginine or any other L-arginine analogs and parameters of the sigmoid model of $\Delta[\text{HHb}]$ responses during exercise (Table 2). Parameters estimated for the sigmoid model of muscle $\Delta[\text{HHb}]$ responses during exercise test are presented in Table 3.

- Table 3 here -

DISCUSSION

The main finding of the present study was that plasma ADMA concentrations were not significantly different between athletes with high exercise capacity (HI-FIT) and healthy subjects with low exercise capacity (LO-FIT). Exercise capacity was previously shown to be negatively associated with ADMA concentrations in patients with chronic heart failure (Seljeflot *et al.*, 2011), peripheral arterial disease (Wilson *et al.*, 2010), in subjects with increased cardiovascular risks (Deftereos *et al.*, 2014) and with aging (Tanahashi *et al.*, 2014b). These previous studies therefore suggested a role for ADMA in the regulation of muscle perfusion, O_2 delivery, and ultimately $\dot{V}O_{2\max}$. However, the negative association between exercise capacity and ADMA has only been reported in pathological conditions and aging, which are characterized by a decrease in exercise capacity. At the other end of the spectrum the high exercise capacity of endurance trained athletes that we compared to healthy

but untrained subjects offered the opportunity to investigate the association between exercise capacity and ADMA at an expanding range of $\dot{V}O_{2\max}$ and without the potentially confounding effects of pathological conditions and aging. Despite $\dot{V}O_{2\max}$ being 60% higher in the HI-FIT group than in the LO-FIT group, the lack of differences for ADMA between the two groups suggests that ADMA is not a key factor regulating O_2 delivery, and consequently O_2 utilization during exercise.

A second finding of the present study was that plasma ADMA concentrations were not associated with the pattern from the sigmoid model of muscle deoxygenation during exercise. Increased O_2 extraction can partly compensate for impaired muscle blood flow during exercise (DeLorey, 2005). Hence, pursuant to the model of Ferreira *et al.* (2007), we initially reasoned that any impairment in endothelium dependent vasodilation caused by ADMA would have resulted in a shift to the left of the sigmoid muscle $\Delta[\text{HHb}]$ profile plotted against the percentage of $\dot{V}O_{2\max}$. The pharmacological increase of ADMA concentrations to patho-physiologically relevant levels ($2.6 \pm 0.3 \mu\text{mol/L}$) has been shown to significantly increase systemic vascular resistance and impair cardiac output in response to exercise (Achan *et al.*, 2003). In a large population of young adults (24-39 years), brachial artery flow-mediated dilation is associated with plasma ADMA concentrations (Juonala *et al.*, 2007), which are within the normal range ($0.29\text{-}0.63 \mu\text{mol.L}^{-1}$, (Blackwell *et al.*, 2007). Moreover, an increase in serum ADMA levels following flow-mediated dilation assessment is associated with a transient endothelial dysfunction during repeated assessment in young healthy male subjects (26 ± 6 years) (Nerla *et al.*, 2011). These studies suggest that ADMA could impair NO-dependent endothelial function in young subjects without pathological conditions. However, our results showed that interindividual differences in plasma ADMA concentrations within the normal range do not appear to significantly affect muscle O_2 delivery and extraction. Hence, ADMA may impair NO-mediated flow dependent vasodilation as previously demonstrated in healthy subjects (Juonala *et al.*, 2007; Nerla *et al.*, 2011) or at low cardiovascular risk (Ardigo *et al.*, 2007), without necessarily affecting the hemodynamic response to exercise.

Together, our results indicate a lack of association of ADMA and any of the L-arginine analogs concentrations with the ability for O_2 muscle delivery and extraction in healthy subjects. These results provide additional support to the hypothesis that, in healthy subjects, interindividual differences in $\dot{V}O_{2\max}$ may not be related to any differences in endothelium-dependent vasodilation (Montero *et al.*, 2014, 2015). Indeed, exercise capacity is significantly associated with endothelial function in less than 50% of studies in healthy subjects after adjustment for age (Montero, 2015). Furthermore, arterial remodeling, especially increase in arterial lumen diameter, may be a more important factor to explain the ability to increase blood flow and O_2 delivery in subjects with high exercise capacity (Green *et al.*, 2004, 2012).

It should be remembered that the contribution of NO to the regulation of bulk limb blood in response to muscle contraction remains controversial (Clifford & Hellsten, 2004). Previous studies fail to show reduced skeletal muscle blood flow during exercise in humans following the infusion of NOS inhibitors (Rådegran & Saltin, 1999; Bradley *et al.*, 1999). Nevertheless, after the pharmacological blockade administered during contraction, NO and prostaglandins independently contribute to exercise hyperaemia (Schrage *et al.*, 2004), highlighting the concept of redundancy in the control of blood flow during continuous exercise. In this study, NO seems to be responsible for 20% of increasing blood flow during rhythmic forearm exercise. In other studies, the double blockade of NO and prostaglandins reduces skeletal muscle blood flow and limits aerobic

metabolism during leg dynamic exercise in young healthy subjects (Mortensen *et al.*, 2007; Christensen *et al.*, 2013). Therefore, ADMA can only impair O₂ delivery into skeletal muscles in conditions where redundant mechanisms of blood flow control during exercise may not be up-regulated to compensate a reduction in NO-dependent pathways (Boushel & Kjaer, 2004). But further research is required to elucidate the precise mechanisms implicated in the regulation of muscle hyperaemia.

Under pathological conditions, the L-arginine to ADMA ratio is a factor that determines NOS activity more strongly than plasma ADMA concentrations alone (Bode-Böger *et al.*, 2007). Low values of the ratio between homoarginine, an alternative substrate of NO synthesis, and ADMA have recently emerged as another marker of cardiovascular risks (Atzler *et al.*, 2013; Tsikas & Kayacelebi, 2014; Kayacelebi *et al.*, 2014). The differences that we observed for these two ratios between the HI-FIT and LO-FIT groups, as well as their weak correlations with $\dot{V}O_{2\max}$, can be explained by at least three hypotheses. First, endurance training stimulates muscle protein turnover (Gibala, 2007); increased protein degradation caused by endurance training in the HI-FIT group may explain the trend for higher ADMA concentrations in the HI-FIT group than in the LO-FIT group in the absence of significant differences between the two groups for any of the L-arginine analogs. Second, changes in the expression and/or activity of cationic amino acids transporters responsible for the ADMA and L-arginine cellular influx and efflux could be altered with endurance exercise training (Shan *et al.*, 2013), and contribute to differences in exchange dynamics of some of the L-arginine analogs between the two groups. Third, the higher homoarginine to ADMA ratio in the LO-FIT group may reduce the level of ADMA by inhibiting the action of protein arginine methyltransferases, and may preserve the L-arginine pool for NO synthesis by inhibiting arginase activity (Michel, 2013). Nevertheless, the effect of exercise on the exchanges of L-arginine analogs between the intra- and extracellular compartments require further research (Tanahashi *et al.*, 2014a). It is to note that despite lower ratios in the HI-FIT relative to the LO-FIT group, being somewhat unexpectedly, the L-arginine/ADMA values were within the range of values reported in healthy subjects (54.3-227) (Bode-Böger *et al.*, 2007). Undisrupted balance between substrate and inhibitor of NO synthesis may contribute to explain the lack of differences in muscle deoxygenation patterns, despite the large difference in $\dot{V}O_{2\max}$ between the two groups.

LIMITATIONS

There are several limitations to the present study. First, plasma nitrate and nitrite concentrations, which are commonly used to reflect NO synthesis, were not measured. However, NO bioavailability, and therefore plasma nitrate and nitrite concentrations, can be significantly increased by factors such as the intake of nitrate-rich food items (Lundberg *et al.*, 2008), or decreased by factors such as the use of antiseptic mouth wash (Pettersson *et al.*, 2009). Plasma nitrate and nitrite thereby do not accurately reflect endogenous NO production. Second, there are limitations related to the NIRS method used to investigate microvascular function in the working skeletal muscle. The NIRS signals reflect changes in HbO₂ and HHb in vessels below 1mm diameter such as arterioles, capillaries and venules, but not in higher caliber arteries where NO-dependent vasodilation can occur (Laughlin *et al.*, 2003a, 2003b; Green *et al.*, 2004); and interindividual differences in the muscle depth penetration of the NIR light can also affect the amplitude of the HHb signal. However, HHb and

$\dot{V}O_{2\max}$ values were normalized relative to their maximal amplitude to minimize the influence of differences in the depth of penetration of the NIR light through the muscle (Boone *et al.*, 2009). The normalization of HHb and $\dot{V}O_2$ also allowed to analyze the pattern of muscle deoxygenation relative to the muscle O_2 demand, independently of the large differences in $\dot{V}O_{2\max}$ between the two groups as previously reported by Boone *et al.* (2010). Third, we did not consider potential plasma volume changes that could have occurred during exercise. However, this factor is unlikely to affect our interpretation of the results since trained and untrained male subjects were reported to have no difference in plasma volume responses following maximal exercise test (Freund *et al.*, 1987).

CONCLUSION

In contrast to previous studies conducted in populations with cardiovascular diseases or increased cardiovascular risks, interindividual differences in exercise capacity are not related to plasma ADMA concentrations or any other L-arginine analogs within a population of young male healthy subjects. Exercise induced-vascular remodeling rather than enhanced NO-dependent vasodilation may contribute to the enhanced muscle perfusion and O_2 delivery in endurance trained athletes. The present findings indicate that plasma ADMA concentrations are unlikely to be a crucial factor contributing to local muscle perfusion and subsequently exercise capacity in healthy male subjects.

AUTHOR'S CONTRIBUTIONS

J.A., J.B., F.-X.G., E.H., and S.B. conceived and designed research; M.P.-C., J.A., J.B., D.M., S.T., F.-X.G., G.C., E.H., J.-F.G., and A.D. performed the experiments and analysis; M.P.-C., J.A., J.B., D.M., S.T., G.C., E.H., J.-F.G., A.D., and S.B. analyzed data; M.P.-C., J.A., J.B., D.M., S.T., F.-X.G., G.C., E.H., J.-F.G., A.D., and S.B. interpreted the results of experiments; M.P.-C. and J.A. prepared the figures; M.P.-C., J.A., J.B., F.-X.G., E.H., and S.B. drafted the manuscript; J.A., J.B., D.M., S.T., G.C., J.-F.G., and A.D. edited and revised the manuscript. All authors approved the final version of manuscript.

CONFLICTS OF INTEREST

The authors do not have any conflicts of interest to disclose.

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TABLES

TABLE 1: Subjects characteristics ^a

	Groups		
	HI-FIT (n = 10)	LO-FIT (n = 10)	
$\dot{V}O_{2\max}$ (mL·min ⁻¹ ·kg ⁻¹) #	70.2 (68.0–71.9)	43.8 (34.8–45.3)	*
Peak aerobic power (W·kg ⁻¹) #	5.3 (4.9–5.5)	3.4 (2.8–3.5)	*
Maximal HR (beats·min ⁻¹) #	180.0 (179.3–187.0)	188.0 (182.5–190.8)	
Age (years)	25.6 ± 7.0	27 ± 6.2	
Body mass (kg)	67.9 ± 9.7	77.2 ± 7.9	
Height (cm)	178.7 ± 8.0	181.0 ± 4.4	
BMI (kg·m ⁻²)	21.2 ± 1.9	23.5 ± 2.0	*
Body fat (%)	10.3 ± 1.7	15.4 ± 3.0	*

^a Values are means ± SD. #: Non-normally distributed variables are presented as median and interquartile range (25th–75th). HI-FIT: high exercise capacity; LO-FIT: low exercise capacity; BMI: Body Mass Index; HR: Heart Rate. *: p < 0.05.

TABLE 2: Spearman's rho and Pearson correlation coefficients between $\dot{V}O_{2\max}$ and parameters of muscle deoxygenation patterns as dependent variables, and L-arginine analogs and their ratios as independent variables ^b

	BEFORE EXERCISE			AFTER EXERCISE		
	$\dot{V}O_{2\max}$	<i>d</i> #	<i>c/d</i> #	$\dot{V}O_{2\max}$	<i>d</i> #	<i>c/d</i> #
ADMA (μM)	0.30	-0.18	-0.02	0.33	-0.08	-0.13
L-arginine (μM)	-0.27	0.15	0.21	-0.40	0.05	0.05
Homoarginine (μM)	-0.32	0.06	0.03	-0.48 *	0.10	-0.16
SDMA (μM)	0.33	-0.26	-0.12	0.14	-0.12	-0.11
L-arginine/ADMA	-0.47 *	0.19	0.24	-0.58 *	-0.04	0.17
Homoarginine/ADMA	-0.43	-0.03	0.19	-0.56 *	0.05	-0.05

^b #: Spearman's rho associations. ADMA: Asymmetric dimethylarginine; SDMA: Symmetric dimethylarginine; *d*: the slope of the sigmoid model; *c/d*: the x value corresponding to 50 % of the total amplitude. *: $p < 0.05$.

TABLE 3: Parameters and goodness-of-fit of the sigmoid model for the muscle deoxygenation ([HHb]) response in the HI-FIT and LO-FIT groups during the maximal exercise test ^c

	Groups	
	HI-FIT	LO-FIT
Parameter estimates		
f_0	-10.7 (-49.1-19.7)	-26.8 (-33.8-13.8)
A	143.8 (86.8-180.0)	137.6 (98.4-158.3)
c	4.6 (2.7-11.4)	4.6 (2.8-7.2)
d	0.09 (0.05-0.18)	0.08 (0.06-0.13)
c/d	60.3 (47.7-69.9)	51.6 (50.3-69.3)
Projected peak	117.2 (106.5-132.7)	113.3 (109.1-115.3)
Goodness-to-fit		
RSS	3471.6 (1356.9-4863.3)	3446.1 (2538.8-6107.5)
R ²	0.97 (0.90-0.99)	0.98 (0.96-0.99)
SEE	5.9 (3.7-7.0)	5.9 (5.0-7.8)

^c Values are median and interquartile range (25th–75th). f_0 : the baseline of % Δ [HHb]_{max}; A : the amplitude of % Δ [HHb] response; c : the constant related to d ; d : the slope of the sigmoid model; c/d : the x value corresponding to 50 % of the total amplitude; Projected peak: sum of $f_0 + A$; RSS: residual sum of squares; R²: coefficient of determination; SEE: the standard error of estimate.

FIGURES

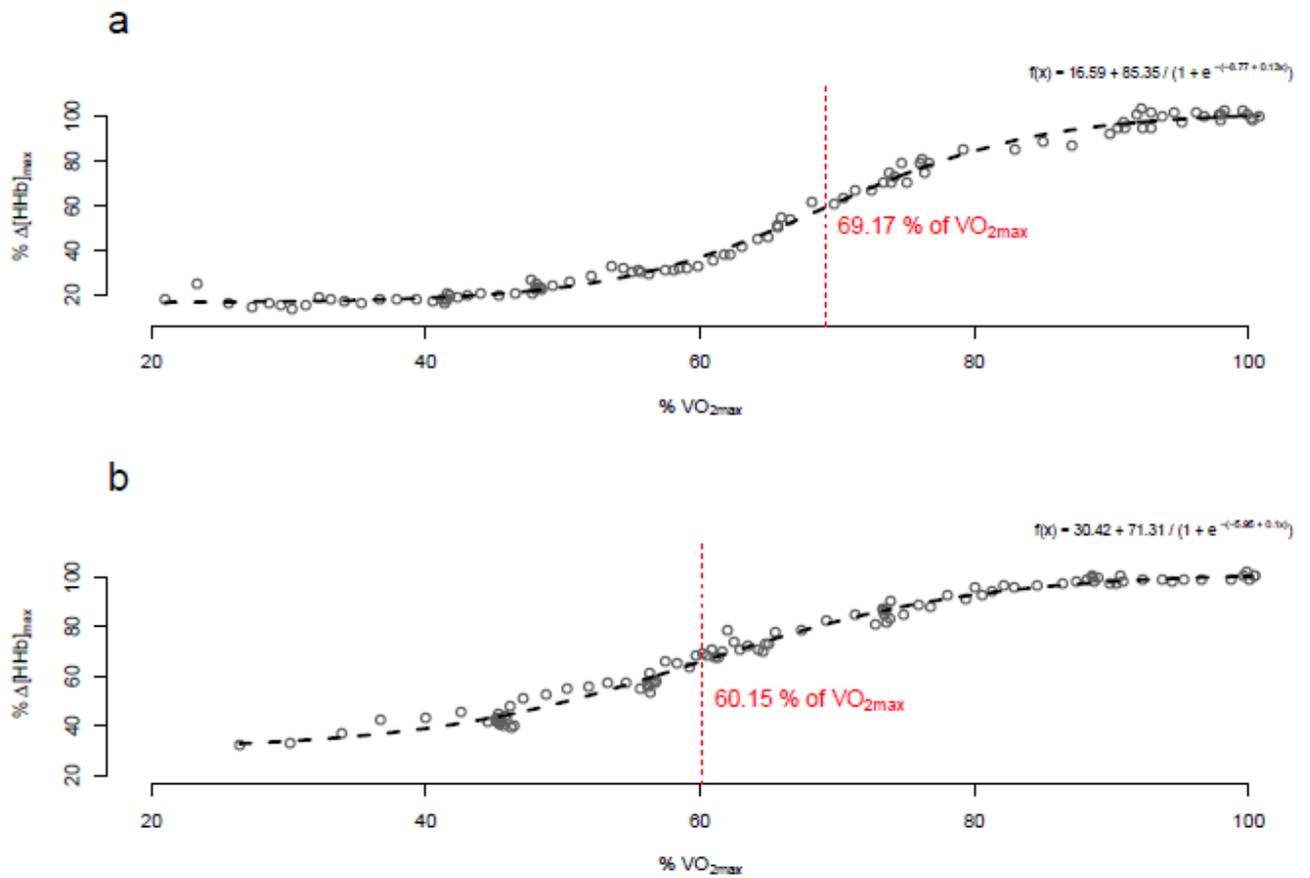


FIGURE 1: Profiles of muscle deoxygenation from two representative subjects in the LO-FIT group (panel a) and in the HI-FIT group (panel b). % $\dot{V}O_{2max}$: percentage of maximal oxygen uptake; % Δ [HHb]_{max}: percentage of change in maximal [HHb] amplitude from baseline. The vertical dashed lines show x-values (% $\dot{V}O_{2max}$) corresponding to 50 % of [HHb] total amplitude.

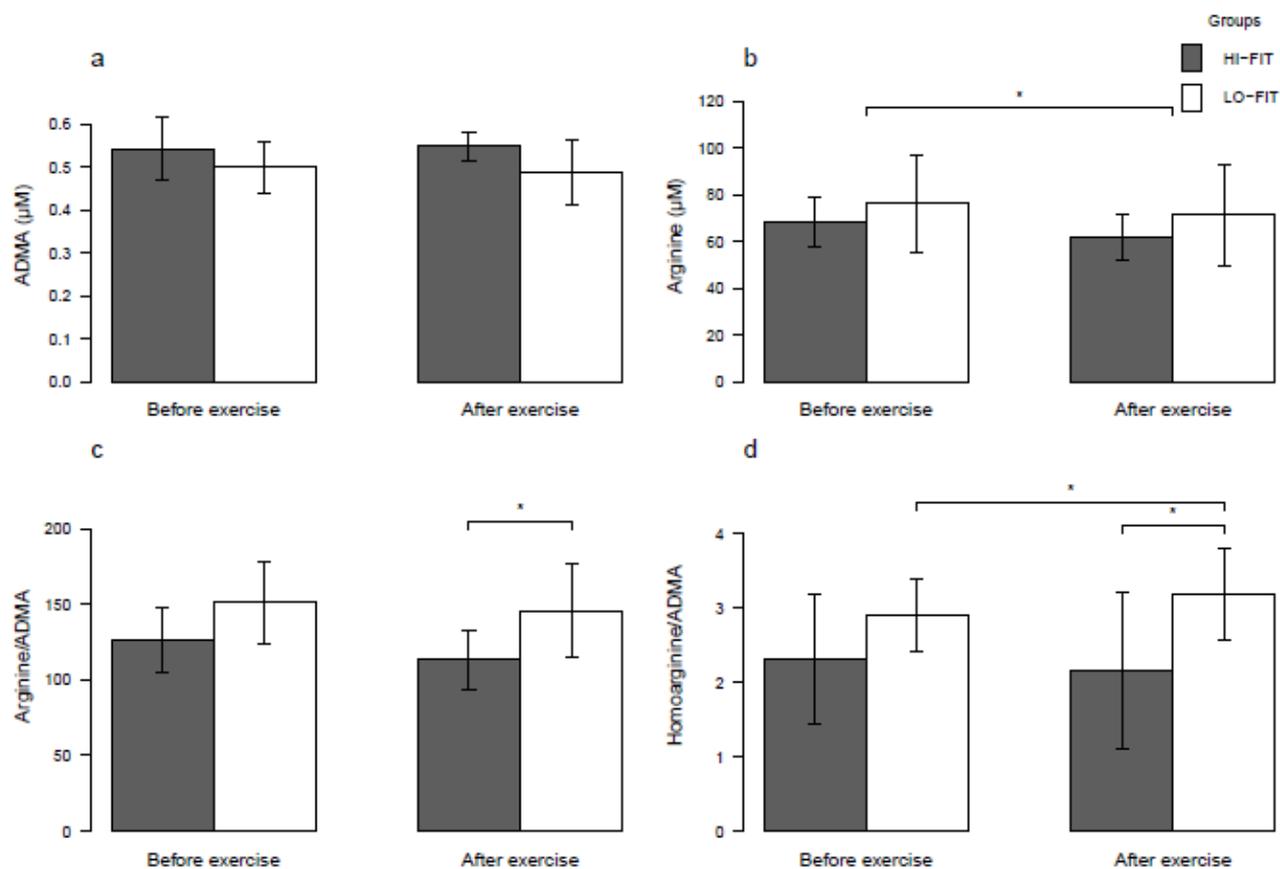


FIGURE 2: Plasma concentrations of ADMA and L-arginine; L-arginine to ADMA ratio; and homoarginine to ADMA ratio for the LO-FIT and HI-FIT groups before and after the maximal exercise test

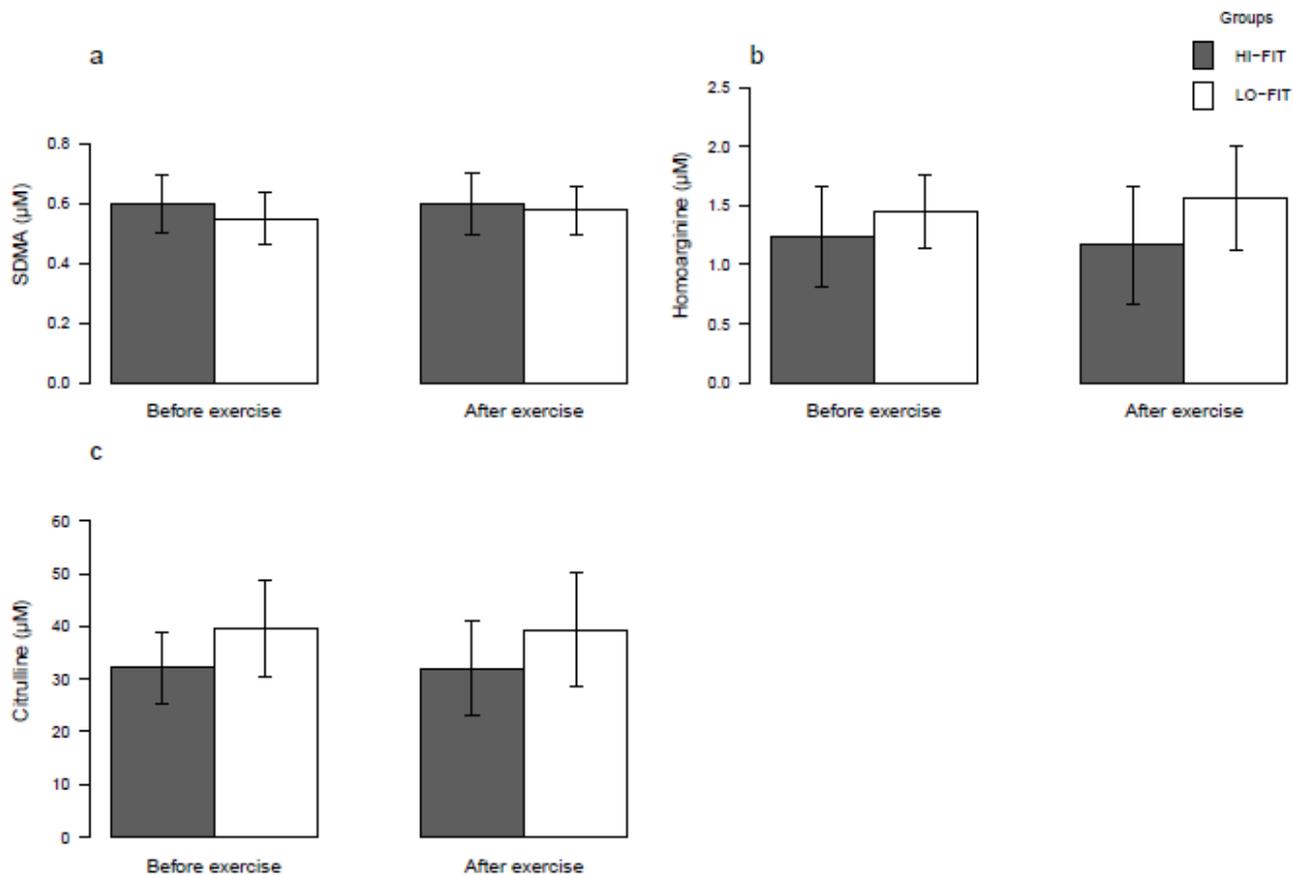


FIGURE 3: Plasma concentrations of Symmetric Dimethylarginine (SDMA), Homocysteine and Citrulline for the LO-FIT and HI-FIT groups before and after the maximal exercise test