Diabetic nephropathy : pathology, genetics and carnosine metabolism

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VEGETARIANISM, FEMALE GENDER AND INCREASING AGE, BUT NOT *CNDP1* GENOTYPE, ARE ASSOCIATED WITH REDUCED MUSCLE CARNOSINE LEVELS IN HUMANS

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Abstract

Carnosine is found in high concentrations in skeletal muscles, where it is involved in several physiological functions. The muscle carnosine content measured within a population can vary by a factor 4. The aim of this study was to further characterize suggested determinants of the muscle carnosine content (diet, gender and age) and to identify new determinants (plasma carnosinase activity and testosterone).

We investigated a group of 149 healthy subjects, which consisted of 94 men (12 vegetarians) and 55 women. Muscle carnosine was quantified in M. soleus, gastrocnemius and tibialis anterior using magnetic resonance proton spectroscopy and blood samples were collected to determine *CNDP1* genotype, plasma carnosinase activity and testosterone concentrations.

Compared to women, men have 36%, 28% and 82% higher carnosine concentrations in M. soleus, gastrocnemius and tibialis anterior muscle respectively, whereas circulating testosterone concentrations were unrelated to muscle carnosine levels in healthy men. The carnosine content of the M. soleus is negatively related to the subjects' age. Vegetarians have a lower carnosine content of 26% in gastrocnemius compared to omnivores. In contrast, there is no difference in muscle carnosine content between subjects with a high or low ingestion of beta-alanine within an omnivore diet. Muscle carnosine levels are not related to the polymorphism of the *CNDP1* gene nor to the enzymatic activity of the plasma carnosinase.

In conclusion, neither *CNDP1* genotype, nor the normal variation in circulating testosterone levels affect the muscular carnosine content, whereas vegetarianism, female gender and increasing age are factors associated with reduced muscle carnosine stores.

Introduction

Carnosine (β-alanyl-L-histidine) is a dipeptide found in high concentrations in skeletal muscles. Several of carnosine's physiological actions are relevant to muscular function and homeostasis, such as pH buffering (1; 2; 5; 6), antioxidant effects (6; 24), increasing the Ca^{2+} sensitivity of the contractile apparatus (12; 25) and inhibiting protein glycation (19), as recently reviewed (6; 8; 37). Interestingly, recent studies have shown that elevated muscle carnosine content is associated with attenuated fatigue and improved exercise performance in humans (10; 18; 35; 40; 41; 44; 48). The carnosine content in human muscles usually amounts to 20-30 $mmol \cdot kg^{-1}$ in dry weight (5-8 mM in wet weight), but can easily vary by a factor 3-4 between the highest and the lowest concentrations measured within a population. Yet, the muscle carnosine content is rather constant, as we showed that intra-individual variation in muscle carnosine content is only 9-15% over a 3-month period (3). The MRS-based technique (10) gives us the opportunity to explore, without the need for muscle biopsy, existing and new determinants of the variation in muscle carnosine content within a large population.

The most established determinant of the muscle carnosine content is muscle fiber type. HPLC-based single fiber analysis in humans showed a 30-100% higher carnosine content in fast-twitch muscle fibers in comparison with slow-twitch (15; 18; 23). Indeed, Mannion *et al.* (27) and Suzuki *et al.* (43) showed a positive correlation between muscle fiber type and muscle carnosine content. Furthermore, elite sprinters who are characterized by a high proportion of fast-twitch muscle fibers have a higher muscle carnosine content in comparison with marathon runners (32).

The amount of food from animal sources is a likely determinant of muscle carnosine levels since beta-alanine, the rate-limiting precursor in carnosine synthesis, is exclusively found in meat and fish. The ingestion of very high doses of beta-alanine in pure form (4-6.4 $g \cdot day^{-1}$ as a food supplement) for several weeks (4-10 wks) results in 40-80% increases in muscle carnosine content (3; 10; 17; 18; 23). Whether variations in daily meat intake within a normal omnivorous diet also affect muscular carnosine content, remains to be established. The chronic and complete withdrawal of dietary beta-alanine, such as in vegetarianism, supposedly results in decreased carnosine content, although the current evidence is scarce (16).

Men have been shown to have higher (21%) carnosine content in the quadriceps femoris when compared to women (26). This sexual dimorphism is more pronounced in mice with a male/female ratio of approximately 3.5/1 (33), but absent in horses (28). Concerning the effect of age, several studies on rodents demonstrated a decreasing muscle carnosine content of 35-50% with senescence (9; 21; 42). To our knowledge, no longitudinal studies on humans are available, but there is cross-sectional evidence for a decreased muscle carnosine content of 33-60% in elderly people with specific pathologies (42; 45).

A possible explanation for a lower muscle carnosine content amongst elderly people and women is their lower plasma (free) testosterone content. Both cross-sectional and longitudinal studies have confirmed an age-associated decline of plasma testosterone in aging men (reviewed in (22). Penafiel *et al.* (33) hypothesized that androgens might up regulate carnosine synthase, based upon the findings that the muscle carnosine content was reduced by 40% in castrated mice and that testosterone injections increased muscle carnosine content by 268% in female mice. To our knowledge, no study has investigated a possible connection between circulating testosterone and muscle carnosine content in eugonadal men.

Polymorphism in the enzymes involved in the synthesis (carnosine synthase) and hydrolysis (carnosinase) of the dipeptide could also contribute to the muscle carnosine content. Since the highest activity of carnosine synthase is found in skeletal muscles (6), polymorphisms of the gene encoding carnosine synthase are likely to clarify variations in carnosine. As the gene has only recently been identified (11), there are no studies that have examined the effects. A leucine repeat in exon 2 of the *CNDP1* gene, coding for the serum carnosinase enzyme, has been shown to affect carnosinase activity (20; 29) and likely the duration of the presence of carnosine in plasma. The enzyme carnosinase is supposedly not present and/or not active in skeletal muscles (3), but it is reasonable to assume that the plasma carnosinase activity could indirectly affect the muscle carnosine content.

The aim of this study was to further characterize previously reported determinants (diet, gender and age) and to identify new potential determinants (plasma carnosinase activity, *CNDP1* genotype and testosterone) of the human muscle carnosine content. Carnosine was quantified using proton magnetic resonance spectroscopy (^{1}H-MRS), as previously described (3; 10; 31), in the slow-twitch soleus and tibialis anterior and the fast-twitch gastrocnemius muscle, in order to explore the possible interaction of these factors with the muscle fiber type.

Materials and methods

Subjects. As depicted in figure 1, a total of 149 healthy subjects volunteered to participate in this study. The muscle carnosine content of 12 male vegetarians was determined. Vegetarian subjects were either lacto-ovo, or vegan and were required to be vegetarian for a minimum of 8 years (mean ± SD: 15 ± 9.5 yr; range 8-36 yr) prior to the study. Blood samples were obtained from all omnivores (82 males, 55 females) for determination of plasma carnosinase activity and *CNDP1* genotype (Age: mean ± SD: 23.9 ± 7.0, range: 18 - 69 yrs). The muscle carnosine content (38 males, 20 females) and plasma concentration of androgens (38 males) were measured in a subgroup of omnivores, of which 29 male individuals registered their meat and fish consumption during 2 weeks. The mean (± SD) age of the male population (23.9 ± 7.2 yrs, range: 19 - 47 yrs) is lower than the age of the vegetarians (31.3 ± 4.2 yrs, range: 22 - 38 yrs) but not different from the female group (23.8 ± 6.7 yrs, range: 19 - 46 yrs). The data of 19 male omnivore subjects are originating from a previous study (3). The study protocols were approved by the local Ethical Committee (Ghent University Hospital, Belgium) and written informed consent was obtained from all participants prior to the study.

^{1}H-MRS. The carnosine content of 3 skeletal muscles of the lower leg (soleus, gastrocnemius and tibialis anterior) of a subgroup of 70 subjects was measured using ^{1}H-MRS, as previously described (3). The subjects lay in supine position on their back and the lower leg was fixed in a holder with the angle of the ankle at 20° plantar flexion. All the MRS measurements were performed on a 3 Tesla whole body MRI scanner (Siemens Trio, Erlangen) equipped with a knee-coil. Single voxel point-resolved spectroscopy with the following parameters was used: repetition time (TR)= 2000 ms, echo time (TE) = 30 ms, number of excitations = 128, 1024 data points, spectral bandwidth of 1200Hz, and a total acquisition time of 4.24min. The average voxel size of the soleus, gastrocnemius lateralis and tibialis anterior was respectively 40 mm x 12 mm x 29 mm, 40 mm x 12 mm x 29 mm, 40 mm x 14 mm x 27 mm. Following shimming procedures, the linewidth of the water signal was on average 24.4 Hz, 25.5 Hz and 27.6 Hz for soleus, gastrocnemius and tibialis anterior, respectively. A 500-ml spherical container filled with an aqueous solution of 20 mM carnosine (Sigma-Aldrich) was used as an external reference for absolute quantification. The following equation was used to determine the concentration of C2-H (at ~8 ppm) carnosine in vivo:

$$[C_m] = [C_r] \cdot (S_m \cdot V_r \cdot C_{T1r} \cdot C_{T2r} \cdot T_m) / (S_r \cdot V_m \cdot C_{T1m} \cdot C_{T2m} \cdot T_r)$$

Where $[C_m]$ is the carnosine concentration in vivo; $[C_r]$ is the concentration of the external reference phantom (20 mM); S_m and S_r are the estimated signal peak areas of the muscle and reference phantom, respectively, obtained by curve fitting performed in the frequency domain and were also corrected for differences in coil loading between phantom and the muscle; corrected for V_m and V_r, the volumes of the voxels in vivo and in the phantom, respectively; C_{T1m}, C_{T2m}, C_{T1r} and C_{T2r} are correction factors for the T1 and T2 relaxation times in vivo and in the phantom, respectively; T_m and T_r are the temperatures in vivo and in the phantom, respectively. The T1 and T2 relaxation times of in vitro carnosine were measured and were found to be 2616 ± 20 ms and 250 ± 29 ms, respectively. The formulae used to calculate the correction factors are:

C_{T1} = (1-e^(-TR/T1))

C_{T2} = e^(-TE/T2)

For the determination of T1 and T2 relaxation times in vivo 5 healthy subjects (2 males and 3 females; age: 21 to 26 yr) were scanned for the soleus, 5 (3 males and 2 females; age: 21 to 25 yr) for the gastrocnemius and 5 for the tibialis anterior muscle (5 females; 22 to 26 yr). T1 was measured using a TE of 30 ms and TR was set to 1500, 2000, 2500, 3000, 3500, 4000, 5000 and 6000 ms. T2 was measured using a TR of 4000 ms and TE was set to 30, 60, 90, 120, 150 and 200 ms for soleus, 30, 45, 60, 75, 90 and 105 ms for gastrocnemius and 30, 45, 50, 60, 70 and 75 for tibialis anterior muscle. For each measurement 128 averages were acquired. The T1 relaxation times were shown to be 1488 ± 377 ms, 1771 ± 225 ms and 1692 ± 432 ms and T2 relaxation times were 152 ± 28 ms, 106 ± 50 ms an 64 ± 32 ms in soleus, gastrocnemius and tibialis anterior, respectively.

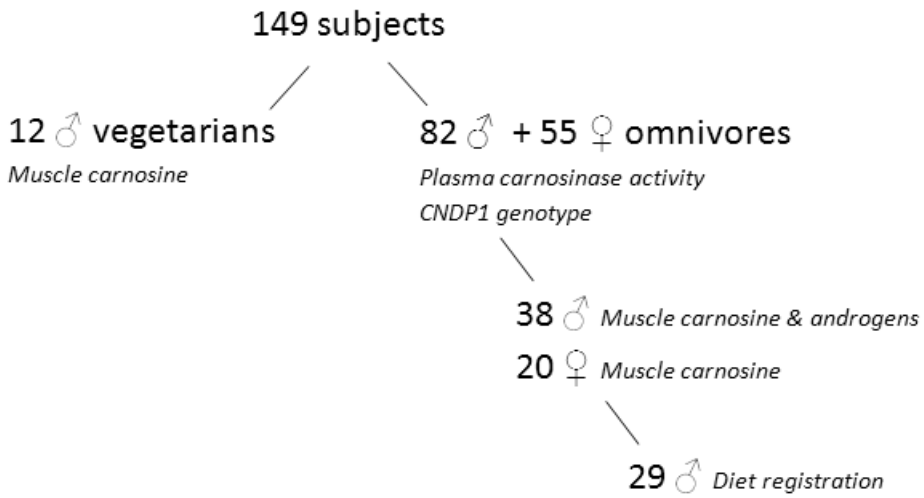

Figure 1. Overview of subject population. Subjects with only plasma are for comparison of *CNDP1* activity between sexes and for relation between *CNDP1* genotype and plasma carnosinase activity

Venous blood sampling. For measurement of circulating androgens, plasma carnosinase activity and for *CNDP1* genotyping, blood of the antecubital vein was withdrawn in heparin tubes. Blood samples were centrifuged and the plasma and blood cells were stored separately at -80°C until further analyses.

Genotyping. A more detailed description of the *CNDP1* genotype determination is explained in the study of Mooyaart *et al.* (29). In brief, a standard PCR protocol was used with primers 5-FAM-GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATT-TGG (reverse). The denaturing, annealing and extension temperatures were 94°C, 60°C and 72°C, respectively. After PCR amplification, fragment analysis was performed on the ABI3130 analyzer (Perkin Elmer) to determine the fragment length corresponding with the different genotypes. Each peak corresponded with the number of leucine repeats on each allele. A 157, 160 and 163 base pair product corresponded with 5, 6 and 7 CTG codons encoding for 5, 6 and 7 leucine repeats, respectively. The 5-5 and the 5-6 *CNDP1* genotypes are widespread and each represent approximately 40% of the population. The 6-6 genotype is present in ± 12% of the population while the 5-7 (± 4%) and 6-7 (± 4%) *CNDP1* genotypes are less common (14; 20; 29).

Plasma carnosinase activity. Heparin plasma samples of 82 men and 55 women were used to quantify the plasma carnosinase activity. Plasma carnosinase activity was

determined according to the method described by Teufel *et al.* (47). Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a plasma sample and stopped after 10 min of incubation at 37°C by adding 1% sulphate salicylic acid. The maximum increase was used for determining the maximum activity. Liberated histidine was derivatized with o-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm. The intra- and inter-assay variations were respectively 7% and 25%. The lowest carnosinase activity detectable was 0.117 µmol/ml/h.

Androgens. Heparin plasma samples of 38 men were analyzed using a commercial immunoassay kit to determine the plasma concentrations of testosterone (Orion Diagnostica, Espoo, Finland) and LH (ECLIA, Roche Diagnostics). The free fraction of testosterone was calculated from plasma testosterone, SHBG, and albumin concentrations using a previously validated equation (49).

Dietary beta-alanine intake. A subgroup of 29 male omnivore subjects completed a questionnaire about their meat and fish consumption during 2 weeks to quantify daily dietary beta-alanine intake, as described in the study of Baguet *et al.* (3).

Statistics. The *CNDP1* genotypes of exon 2 were categorized based on the leucine repeat length (5-5, 5-6, 5-7, 6-6, 6-7). Independent sample T-tests were used to evaluated the effect of gender, age and *CNDP1* genotype on the muscle carnosine content. An univariate analysis of variance with age as covariate was used to verify the effect of vegetarianism on muscle carnosine content. The correlation between genotype and carnosinase activity was assessed by an univariate analysis of variance with carnosinase activity as dependent and both gender and *CNDP1* genotype as independent variables (post hoc: pairwise comparisons). The other possible determinants were analyzed by bivariate correlations. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and a p-value < 0.05 was considered to be statistically significant.

Results

Gender. As illustrated in figure 2A, men had a higher muscle carnosine content in comparison to women ($p \leq 0.004$), but the magnitude of this sex-related difference depends on the type of muscle. The soleus and gastrocnemius showed a 36% and 28% larger carnosine content in men, whereas the difference was 82% in tibialis anterior.

The plasma carnosinase activity was significantly higher ($p < 0.001$) in women (7.20 ± 1.62 µmol/ml/h) in comparison with men (5.79 ± 1.58 µmol/ml/h; figure 2B).

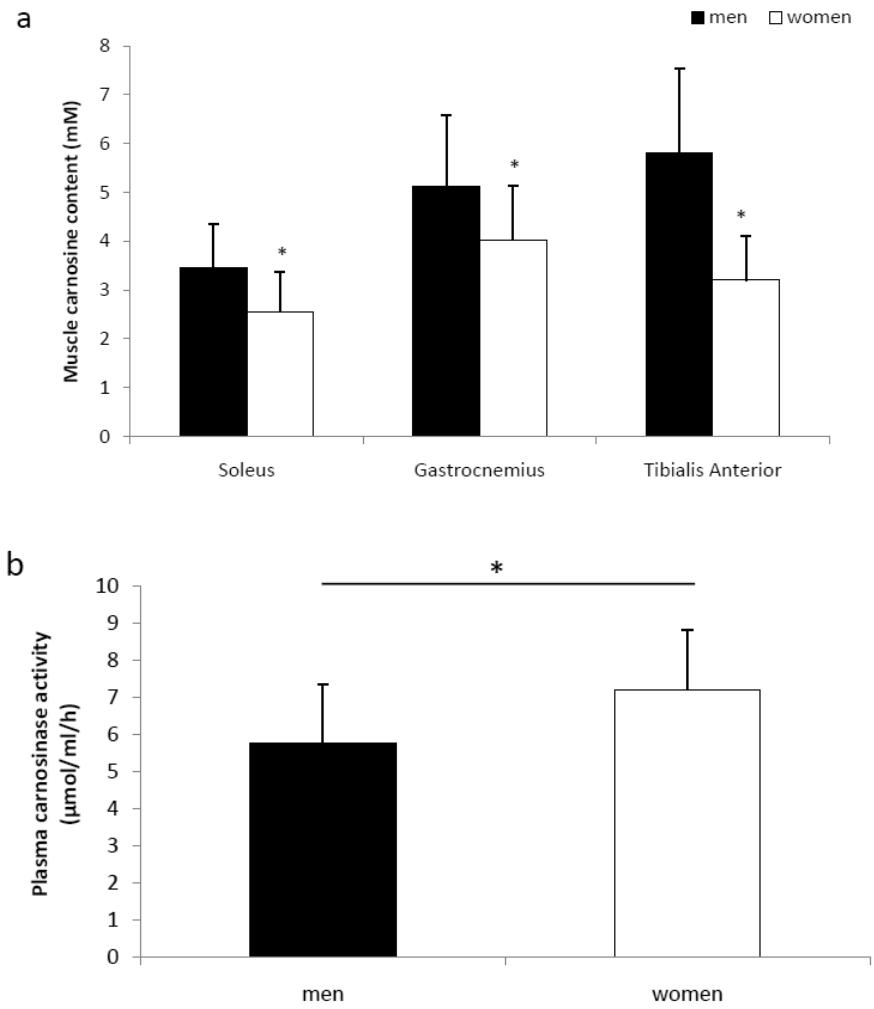

Figure 2. a/ The carnosine content of men (n = 38) versus women (n = 20) in soleus, gastrocnemius and tibialis anterior, measured by proton spectroscopy, * different from men ($p \leq 0.004$). b/ The plasma carnosinase activity of the female (n = 55) is 24,3% higher in comparison with the carnosinase activity of the male population (n = 82) ($p < 0.001$)

Age. The carnosine concentration in the M. soleus (n = 58, p = 0.049; r = -0.260, figure 3) declines with age in the adult range of 19-47 years (M. gastrocnemius: p = 0.112; r = -0.211, M. tibialis anterior: p = 0.482; r = -0.096). In the same group, age did not significantly correlate with plasma carnosinase activity (p = 0.355; r = 0.124).

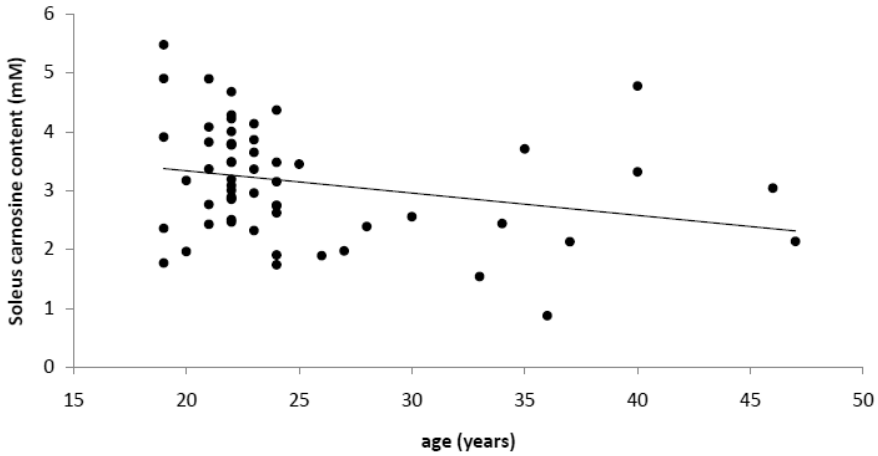

Figure 3. Effect of age on muscle carnosine content in both male and female omnivores. There is a negative correlation of age and carnosine concentration in soleus ($p < 0.05$, $r = -0{,}260$, $n = 58$)

Androgens. In order to elucidate the mechanisms of the age and gender related effects on the muscle carnosine content, we measured plasma testosterone and free testosterone concentrations. The mean (± SD) total testosterone and free testosterone plasma levels in the male reference population were, respectively, 538.6 ± 140.3 $ng \cdot dl^{-1}$ and 11.7 ± 3.5 $ng \cdot dl^{-1}$. Neither of them correlated with muscle carnosine content (soleus, gastrocnemius and tibialis anterior) nor with carnosinase activity ($0.238 \leq p \geq 0.921$). A scatter plot of plasma free testosterone content with M. soleus carnosine content is depicted in figure 4. The plasma total and free testosterone concentration is inversely related to the subjects' age ($p < 0.02$; $r = -0.410, -0.402$; respectively).

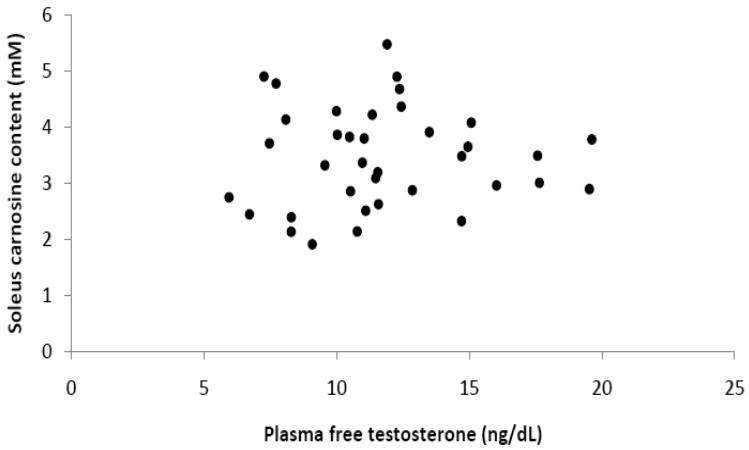

Figure 4. The lack of correlation between plasma free testosterone and soleus carnosine content (p = 0.921). Similar results were obtained for gastrocnemius and tibialis anterior (p = 0.851 and p = 0.794, respectively) and for the correlation of the muscle carnosine content with testosterone, luteinizing hormone (LH) and sex hormone binding globulin (SHBG) (p > 0.05)

Daily beta-alanine intake. Long-term vegetarianism (> 8 years) is associated with declined muscle carnosine stores (figure 5). Vegetarians have lower carnosine levels of 17% in M. soleus (p = 0.05) and 26% in M. gastrocnemius (p = 0.009) and a trend to a lower carnosine content (20%) in M. tibialis anterior (p=0.068) compared to omnivores. However, the significance of the effect of vegetarianism on soleus carnosine content disappeared, when the data were corrected for age (p = 0.304), whereas this was not the case in M. gastrocnemius nor in tibialis anterior. The mean (± SD) daily average beta-alanine ingestion by meat and fish consumption in a subgroup of 29 omnivore male subjects was 0.332 ± 0.144g. Within a normal Western omnivore diet, beta-alanine intake by meat and fish consumption is not a determinant of the muscle carnosine content, as there is no correlation between beta-alanine ingestion and muscle carnosine content ($0.671 \leq p \geq 0.885$) nor a difference in muscle carnosine content between subjects with a low (< 0.332 $g \cdot day^{-1}$) or a high (> 0.332 $g \cdot day^{-1}$) intake of beta-alanine ($0.296 \leq p \geq 0.562$).

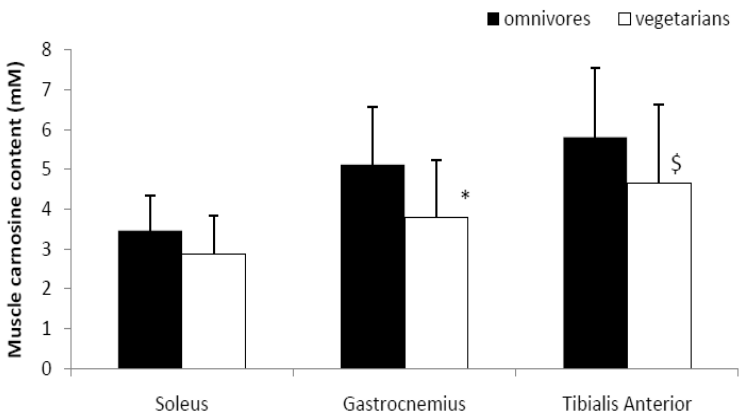

Figure 5. Male vegetarians (n = 12) have a lower muscle carnosine content (* $p < 0.05$ and \$ $p < 0.10$) in comparison with male omnivores (n = 38)

Genotype and plasma carnosinase activity. The most common *CNDP1* genotypes were 5-5 (35.8%) and 5-6 (38%). The 5-7, 6-6 and 6-7 *CNDP1* genotypes were detected in respectively 7.3%, 15.3% and 3.6% of the subjects. Figure 6a shows that the plasma carnosinase activity is dependent on the *CNDP1* genotype ($p = 0.054$). The plasma carnosinase activity of the 5-5 genotype is lower compared to the 5-6 ($p = 0.05$) and to the 6-6 genotype ($p = 0.025$). Also the 6-7 alleles show a lower plasma carnosinase activity than the 6-6 alleles ($p = 0.035$). The relation between the most frequent *CNDP1* genotypes (5-5 and 5-6) and the muscle carnosine content is depicted in figure 6b. The muscle carnosine content of the individuals with the 5-5 *CNDP1* genotype is similar to the carnosine levels of the subjects with the 5-6 *CNDP1* genotype, in both M. soleus, gastrocnemius and tibialis anterior ($0.393 \leq p \leq 0.576$). Likewise, there is no correlation between muscle carnosine levels and the activity of the carnosine degrading enzyme in plasma ($0.154 < p < 0.744$).

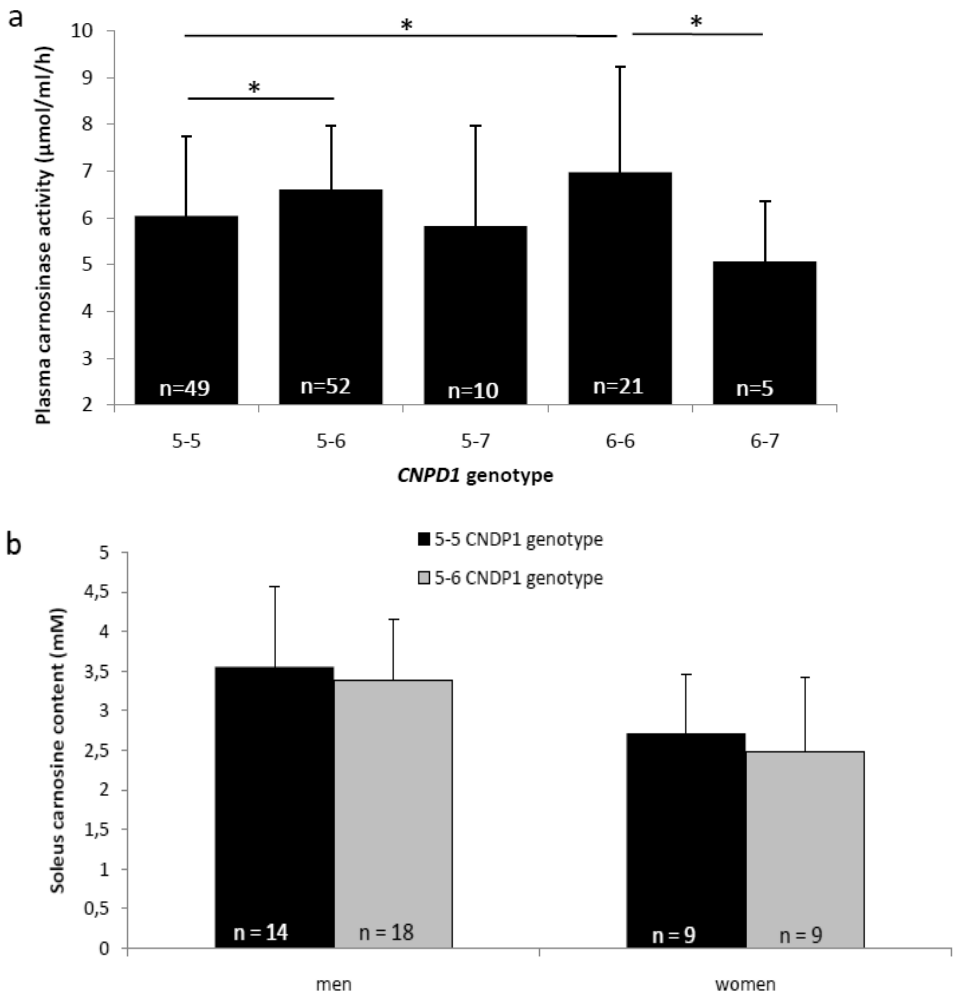

Figure 6. a/ Mean (± SD) plasma carnosinase activity (µmol/ml/h) categorized by amount of leucine repeats in *CNDP1* gene of both 82 males and 55 females. b/ The carnosine content of individuals with the 5-5 *CNDP1* genotype in comparison with individuals with the 5-6 *CNDP1* genotype ($p > 0.05$)

Discussion

Carnosine is synthesized in skeletal muscles from histidine and beta-alanine, with the latter being the rate-limiting precursor. It is demonstrated that an increased availability of beta-alanine, by means of oral supplementation, results in higher muscle carnosine levels (for review see: (8; 37). This is the first peer-reviewed study which shows that a complete and long-lasting restriction of beta-alanine from the diet, as is the case in habitual vegetarians (lacto-ovo or vegan, > 8 yrs), results in lower intramuscular carnosine concentrations as compared with omnivorous subjects. In a proceedings abstract by Harris *et al.* (16), it was shown that female vegetarians have a 45% lower carnosine content in M. vastus lateralis compared with male sports science students. We observed a 26% lower carnosine content in the gastrocnemius of male vegetarians compared to male omnivores. The lower muscle carnosine levels in vegetarians implies that it may be important for vegetarian athletes, involved in high-intensity exercise, to compensate a possible shortage of muscle carnosine by means of beta-alanine supplementation, as also recommended for creatine (13). However, the decreased intramuscular total creatine content in vegetarians versus omnivores is less pronounced, namely 11.1% (7). As there is no difference in muscle carnosine content between individuals with a higher or a lower beta-alanine intake within a normal western omnivore diet, we can conclude that only the supplementation with very high doses of beta-alanine and the complete and prolonged restriction of beta-alanine from the diet does influence the carnosine content.

This study confirms the higher muscle carnosine content in males versus females shown by Mannion *et al.* (26), but in the currently studied muscles of the lower leg, the gender-based differences are even more pronounced (28-82%), compared to the differences in M. quadriceps femoris of the previous study (21%). To the best of our knowledge, no other metabolite in the muscle shows such a large gender-dependent difference. In that light, it is interesting that a sex-specific effect was seen in the relation between diabetic nephropathy and the *CNDP1* genotype (30). Additionally, this gender difference could contribute to the lower high-intensity exercise capacities of women compared to men. Besides the effect of gender on the muscle carnosine content, there is also a negative correlation between age and muscle carnosine content. Stuerenburg *et al.* (42) described a correlation coefficient of -0.4 in patients with neuromuscular diseases ranging from 20 to 80 years. This study is the first that shows this negative

correlation in a healthy adult population (19-47 years). Our cross-sectional data suggest a decline in soleus carnosine content of 1.2%/yr. However, the majority of the subjects are younger than 30 years and the confirmation of these data in an older healthy adult population is recommended. A number of possible factors exist as to why the carnosine levels are affected by gender and age. We hypothesize that both muscle fiber type and circulating androgen levels could be responsible for this gender and age based distinction in muscle carnosine levels.

Despite the conflicting reports regarding the percentage muscle fiber type proportion amongst gender and age, it is generally acknowledged that women and elderly people are characterized with a smaller cross-sectional area of muscle fibers which is most pronounced in fast-twitch fibers (38; 39). Women have 56% smaller cross-sectional area (CSA) of type IIB/X muscle fibers in comparison with men, whereas this gender-induced difference is only 14% in slow-twitch muscle fibers (38; 39). As already mentioned in the introduction, fast twitch muscle fibers are characterized by 30 - 100% higher carnosine levels compared to slow-twitch muscle fibers (15; 18; 23). Thus, it is possible that women and elderly people have lower carnosine levels as a result of a smaller proportion of fast twitch muscle fibers compared to young men.

The hypothesis that circulating androgen levels could affect the muscular carnosine levels is based on the study of Penafiel *et al.* (33) in which they successfully manipulated the carnosine content of murine skeletal muscles by means of subcutaneous testosterone injections in female mice and by removing the testes in male mice. However, we found no correlation between the plasma (free) testosterone levels and muscle carnosine content within a healthy male population. Nevertheless, this does not exclude the possibility that more extreme variations in total or free testosterone, such as overt hypogonadism or exogenous testosterone supplementation do influence muscle carnosine content. This argumentation is supported by the two-fold higher muscle carnosine content of the bodybuilders in the study of Tallon *et al.* (46), in which 5 of the 6 subjects reported to have taken anabolic steroids in the last 6 months.

The observed influence of vegetarianism, gender and age on carnosine content seems to depend on the muscle type under investigation. The gastrocnemius muscle is most affected by dietary beta-alanine restriction, the tibialis anterior is the muscle that displays the largest sexual dimorphism and the age-related effect on muscle carnosine content is only observed in soleus. Muscle-specific differences in expression profiles of e.g. enzymes of carnosine metabolism, amino acid transporters or androgen receptors could be hypothesized.

In line with previous reports, we observed that the *CNDP1* polymorphism affects the plasma carnosinase activity. The higher activity of the carnosine degrading enzyme for the 5-6 and the 6-6 *CNDP1* genotypes compared to the homozygosity for the Mannheim allele (5-5) is confirmed (20; 29). However, it has to be noted that there is a high variation in plasma carnosinase activity within one genotype group (e.g. a range of 2-10 µmol/ml/h in 5-5 genotype). Remarkably, individuals with the 6-7 *CNDP1* genotype have a significantly lower carnosinase activity than individuals with the 6-6 variant. This is in contrast with the suggestion of Janssen *et al.* (20) that both the 6 and 7 leucine alleles can be regarded as gain-of-function mutations associated with a higher enzyme activity and with the results of Riedl *et al.* (36) that the percentage of secreted carnosinase increases with increasing length of the leucine repetitions in the signal peptide. Variations of natural inhibitors of carnosinase such as homocarnosine may account for additional fluctuations in enzyme activity (34). Furthermore, the results of this study reveal that females are characterized with a higher plasma carnosinase activity versus males, confirming and strengthening the non-significant gender-based differences in plasma carnosinase levels which were previously reported (4; 34). Despite the combination of declined muscle carnosine levels and a higher activity of the plasma carnosinase enzyme in both women and elderly people (34), we shown no inverse relationship between both parameters in a gender - and age - mixed population. Similarly, also the polymorphism of the *CNDP1* gene does not determine the muscle carnosine levels. A different compartimentation could underlie this finding, as carnosine is mainly present in the muscle and the enzyme carnosinase is present in the circulation but not (active) in the muscle. Additionally, muscle carnosine could be more sensitive to the carnosine synthetase activity than to the carnosinase activity. However, we are aware that our population is probably too small to completely exclude the possibility that muscle carnosine is related to *CNDP1* genotype. It is therefore necessary to confirm these data in a larger population with a relevant number of subjects with less common genotypes (5-7, 6-6, 7-7).

In conclusion, the results of this study provide evidence for (I) a declined muscle carnosine content in vegetarians which implies that it may be important for vegetarian athletes, involved in high-intensity exercise, to compensate a possible shortage of muscle carnosine by means of beta-alanine supplementation, (II) a marked sexual dimorphism in muscle carnosine levels and plasma carnosinase activity and (III) an independency of the muscle carnosine content to the polymorphism of the *CNDP1* gene and the enzymatic activity of plasma carnosinase.

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The authors declare that they have no conflict of interest.

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