Low plasma carnosinase activity promotes carnosinemia following
carnosine ingestion in humans

Running title: Supplementation-induced carnosinemia in humans

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Abstract

Objectives A polymorphism in the CNDP1 gene, resulting in decreased plasma carnosinase activity, is associated with a reduced risk for diabetic nephropathy. Because carnosine, a natural scavenger/suppressor of reactive oxygen species, AGE’s and reactive aldehydes, is readily degraded in blood by the highly active carnosinase enzyme, it has been postulated that low serum carnosinase activity might be advantageous to reduce diabetic complications. The aim of this study was to examine whether a low carnosinase activity promotes circulating carnosine levels following carnosine supplementation in humans.

Methods Blood and urine was sampled, in 25 healthy subjects following acute supplementation with 60mg/kg BW carnosine. Pre-cooled EDTA tubes were used for blood withdrawal and plasma samples were immediately deproteinized and analyzed for carnosine and beta-alanine by HPLC. CNDP1 genotype, baseline plasma carnosinase activity and protein content were assessed.

Results Upon carnosine ingestion, 8 of the 25 subjects (= responders) displayed a measurable increase in plasma carnosine up to 1h following supplementation. Subjects with no measurable increment in plasma carnosine (= non-responders) had an approximately 2-fold higher plasma carnosinase protein content and an approximately 1.5-fold higher activity compared to the responders. The urinary carnosine recovery was 2.6-fold higher in the responders versus non-responders and was negatively dependent on both the activity and protein content of the plasma carnosinase enzyme.

Conclusion A low plasma carnosinase activity promotes the presence of circulating carnosine upon an oral challenge. These data may further clarify the link between CNDP1 genotype, carnosinase and diabetic nephropathy.

Keywords CNDP1, beta-alanine, diabetic nephropathy
**Introduction**

Adequate glycemic and blood pressure control are the most effective therapeutic modalities in diabetic patients to delay the onset of microvascular complications (1). Nonetheless, an increasing number of patients will continue to develop microvascular complications despite these therapeutic measures. Ample evidence indicates that susceptibility to develop diabetic nephropathy, the most common cause of renal failure in the western world, is genetically determined (23). One of the genes that have recently been linked to diabetic nephropathy is carnosinase dipeptidase-1 (CNDP1), encoding the serum carnosinase enzyme (16). Diabetic nephropathy is strongly associated with a (CTG)$_n$ polymorphism in the CNDP1 gene, affecting serum carnosinase secretion (30). Diabetic patients homozygous for (CTG)$_5$ have a lower risk to develop diabetic nephropathy and have a lower plasma carnosinase activity (16). Although the association between the CNDP1 polymorphism and diabetic nephropathy have been confirmed in an independent study in European Americans (11), other studies did not show an association in type 1 diabetic patients (37) or showed that the association in type 2 diabetic patients is sex specific (25). Inconsistent findings may be explained by differences in ethnicity (22) or alternatively, by assuming that protection from diabetic nephropathy afforded by (CTG)$_5$ homozygosity in CNDP1 may be masked by additional risk haplotypes (9; 22).

Glycation and oxidative stress (glycoxidative stress) and accelerated formation of advanced glycation end-products (AGE’s) during hyperglycemia are implicated in the development of diabetic complications (8). Carnosine (β-alanyl-L-histidine) is a versatile dipeptide, mainly present in neuronal tissue and skeletal muscle, that has the propensity to suppress several aspects of glycoxidative stress, such as inhibition of AGE formation (14), quenching of reactive aldehydes (3) and suppression of oxidative stress (18). Moreover, it has been shown that carnosine inhibits...
angiotensin converting enzyme (ACE) (15), albeit only at high concentrations. The influence of
carnosine on blood pressure control has thusfar not been demonstrated.

In humans, circulating carnosine is readily degraded by the highly active serum carnosinase
enzyme, which is secreted from the liver into the plasma (30). Therefore, plasma carnosine
concentrations in fasted subjects are in general below the detection limits of current quantitative
carnosine assays. Also the presence of carnosine in plasma (carnosinemia) following dietary intake
of pure carnosine or meat, which is a rich source of carnosine, is controversial. Both Asatoor et al.
(5) and Gardner et al. (12) could not detect carnosine in plasma after administration of a high dose
of carnosine (respectively ~ 60mg/kg BW and 4g). Cooling of the samples following blood
withdrawal however, resulted in the detection of a small amount of plasma carnosine in one subject
in the latter study of Gardner et al. (12). Despite the use of EDTA blood tubes, which have been
shown to inhibit the hydrolysis of carnosine in plasma, no plasma carnosine was detected after oral
supplementation with 450mg carnosine (38). In line with this, both Harris et al. (13) and Yeum et
al. (38) could not detect any carnosine in plasma after the ingestion of beef, chicken breast nor
chicken broth. These results are in sharp contrast to the findings of Park et al. (27) who reported a
peak plasma carnosine concentration of 32.7mg/L 3.5h after ingestion of ground beef. The
discrepancy between these studies could be due to differences in blood handling or due to different
carnosinase expression of the volunteers as Yeum et al. (38) used female subjects (40-60 years)
which are characterized by higher carnosinase activity compared to males (10).

The current working hypothesis to explain the physiological mechanism for the protective effect of
(CTG)$_5$ homozygosity puts forward that 1) a $CNDP1$ genetic predisposition leads to low serum
carnosinase activity (16) 2) low carnosinase activity promotes higher concentrations of circulating
carnosine, and 3) high circulating carnosine levels protect against hyperglycemia-induced cytotoxic
metabolites, resulting from oxidative stress and glycation. Evidence for the latter has been provided
in a number of animal studies were carnosine supplementation could delay the development and
progression of diabetes in db/db mice (32) and resulted in reduced urinary markers of oxidative stress and AGE’s in obese Zucker rats (4). There is currently however insufficient experimental evidence that supports the assumption that low carnosinase activity promotes higher concentrations of circulating carnosine. Therefore, the current study aims to explore the impact of variation between humans in carnosinase activity on circulating carnosine concentrations. We hypothesize that higher circulating carnosine concentrations can be detected in subjects with low carnosinase activity, following a single large oral dose of carnosine (60 mg/kg BW).
Methods

Subjects

Twenty-five subjects (age: 20-31 years, body weight: 70.9 ± 9.8 kg), both male (n = 15) and female (n = 10), participated in this study. All subjects were in good health and none of the participants was vegetarian. The study protocol was approved by the local ethical committee (Ghent University Hospital, Belgium) and written informed consent was obtained from all participants prior to the study.

Study design

Heparin plasma was obtained prior to the experiments and on the morning before the supplementation to quantify plasma carnosinase protein content and activity. After an overnight fast (at least 8 h), an indwelling catheter was inserted in an antecubital vein and blood was withdrawn before and 20, 30, 40, 60 and 120 minutes following oral supplementation of 60 mg/kg BW carnosine (mean ± SD: 4209 ± 577 mg), dissolved in 330 ml water. Blood samples for determination of carnosine were collected in pre-cooled (4°C) EDTA tubes and immediately centrifuged (4°C) to separate the plasma. The anticoagulant EDTA was chosen for its ability to chelate Zn^{2+} ions which are essential for the catalytic activity of carnosinase (38). Plasma samples were deproteinized with SSA (35%) and stored immediately at -20 °C until further analysis. Urine was collected in EDTA-coated tubes prior and 45, 90, 135, 180 and 240 minutes after the carnosine supplementation. The subjects were allowed to drink water and received a carnosine-free meal after blood collection.

Determination of carnosine and beta-alanine by HPLC

100 µL of deproteinized EDTA plasma and urine was dried under vacuum (40 °C). Dried residues were resolved with 40 µL of coupling reagent: methanol-triethylamine-H2O-phenylisothiocyanate
(PITC) (7:1:1:1) and allowed to react for 20 minutes at room temperature. The samples were dried
again and resolved in 100 µL of sodium acetate buffer (10 mM, pH 6.4). The same method was
applied to the standard solutions of beta-alanine (Sigma) and carnosine (Flamma, dissolved in
deionized-distilled water). The derivatized samples (20 µL) were applied to a Waters HPLC system
with an Hypersilica column (4.6 x 150 mm, 5 µm) and UV detector (wavelength 210 nm). The
column was equilibrated with buffer A [10 mM sodium acetate adjusted to pH 6.4 with 6 % acetic
acid] and buffer B [60 % acetonitrile – 40 % buffer A] at a flow rate of 0.8 ml/min at room
temperature. Limit of detection and quantification were respectively 3 and 10 µM.

**Determination of carnosine by LC-ESI-MS/MS**

Analyses of plasma carnosine of one subject were performed by using a validated LC-ESI-MS/MS
method (26). Briefly, aliquots of 100 µl of EDTA-plasma samples were spiked with H-Tyr-His-OH
as internal standard (20 µM final concentration), deproteinized by perchloric acid (PCA, 700 mM
final concentration) and centrifuged at 18,000 rpm for 10 min. The supernatants were then diluted
1:1 with mobile phase A (CH3CN/H2O/HFBA 90/10/0.1 v:v:v), filtered through 0.2 µm filters and
then injected into a ThermoFinnigan Surveyor LC system equipped with a quaternary pump and
connected through an electrospray interface (ESI) to a TSQ Quantum Triple Quadrupole Mass
Spectrometer (ThermoFinnigan Italia, Milan, Italy). Chromatographic separations were done by
reverse phase elution with a Phenomenex Sinergy polar-RP column (150 mm x 2 mm i.d.; particle
size 4 µm) (Chemtek Analytica, Anzola Emilia, Italy) protected by a polar-RP guard column (4 mm
x 2 mm i.d.; 4 µm) kept at 25 °C. Separations were done by gradient elution from 100% phase A to
80% phase B (CH3CN) in 12 min at a flow rate of 0.2 ml min-1 (injection volume 10 µl); the
composition of the eluent was then restored to 100% A within 1 min, and the system was re-
equilibrated for 6 min. Quantitations were performed in multiple reaction monitoring (MRM) mode
at 2.00 kV multiplier voltage, and the following MRM transitions of \([M + H]^+\) precursor ion → product ions were selected as follows:

- H-Tyr-His-OH (IS) m/z 319.2 → 156.5 + 301.6 (collision energy, 25 eV);
- CAR: m/z 227.0 → 110.6 + 156.5 (collision energy, 25 eV);

**Determination of plasma carnosinase activity and protein content**

At baseline, the mean plasma carnosinase activity and protein content was quantified based on the activity and protein content of two different blood collections. Plasma carnosinase activity was determined according to the method described by Teufel et al (35). Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a heparin plasma sample and stopped after 10 min of incubation at 37 °C by adding 1 % SSA. Liberated histidine was derivatized with o-phtaldialdehyde (OPA) and the maximum increase was used for determining the maximum activity. 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.

Plasma carnosinase protein content was measured by ELISA. In brief, a human CN1 ELISA was developed by coating high absorbant microtitre plates (Greiner BioChemia, Flacht, Germany) overnight with 100 µl of goat polyclonal anti-human CN1 (1 µg/ml) (R&D, Wiesbaden Germany). The plates were extensively washed and incubated with 5 % w/v of dry milk powder to avoid unspecific binding. For each sample and standard serial dilution were carried out. The plates were placed on a shaker for 1 hr and subsequently extensively washed with PBS/Tween. Hereafter anti-human carnosinase monoclonal antibody (clone ATLAS, Abcam) was added for 1 hr followed by extensively washing. HRP conjugated goat anti-mouse IgG was added for 1 hr and the plates were washed. After addition of peroxidase substrate (deep-Blue POD) (Roche diagnostics, Mannheim, Germany) the reaction was stopped after 15 minutes by addition of 50 µl of 1 M H₂SO₄ and read in
an ELISA reader at 450 nm. CN1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA was approximately 20 ng/ml.

**CNDP1 genotyping**

A more detailed description of the *CNDP1* genotype determination is explained in the study of Mooyaart et al. (24). In brief, a standard PCR protocol was used with primers 5-FAM-GCGGGAGGGTGAGGAGAAC (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.

**Statistics**

Data are expressed as mean ± SD. Bivariate correlations and independent sample T-tests were used for statistical analysis (SPSS 17).
Results

*Carnosine is detectable in human plasma by HPLC.*

A HPLC chromatogram of EDTA plasma derivatized with PITC (UV detection) of a subject 30 minutes following 60 mg/kg BW carnosine ingestion is depicted in Figure 1A. Spiking the sample with a carnosine standard results in an identical chromatogram except for the higher peak at 15.6 min, representing carnosine. If the necessary precautions to block the endogenous carnosinase activity are not taken (heparin instead of EDTA tubes; not pre-cooled; not immediately deproteinized), then the same subject at the same time point does not display a carnosine peak at 15.6 min (Figure 1B) and the peak of beta-alanine (the product of the carnosinase reaction) increases, which illustrates that the peak at 15.6 min genuinely represents carnosine. Plasma samples of one subject were analysed by an independent laboratory with LC-ESI-MS/MS method (26) and carnosine was detected at 30 (11.8µM) and 45 (3.4µM) minutes following carnosine supplementation (4g) while at the other time-points no carnosine could be detected.

*Supplementation-induced carnosinemia depends on CN1 protein content and activity.*

The mean CN1 protein levels varied widely between subjects from 24.38 to 148.02 µg/ml (mean ± SD: 77.82 ± 30.98 µg/ml) and the CN1 activity values were situated between 2.79 and 10.90 µmol/ml/h (mean ± SD: 5.95 ± 1.91 µmol/ml/h). Furthermore, the CN1 protein content is positively correlated to the activity of the enzyme (p = 0.004, r = 0.58, R² = 0.34, Figure 2), confirming that the activity level is largely determined by the amount of enzyme available in the plasma. Upon carnosine ingestion, 8 of the 25 subjects displayed an increase in plasma carnosine concentration (carnosinemia), which we termed as responders (increase of > 10 µM carnosine after supplementation). The increase in plasma carnosine reached its C_{max} (mean ± SD: 73.3 ± 59.7 µM; range: 30.7 - 195 µM) at on average 30 - 40 minutes after supplementation and rapidly declined within 1 - 2 hours (Figure 3). However, the remaining 17 subjects had no measurable increment (=
non-responders) in plasma carnosine after oral supplementation with a high dose of carnosine, despite the precautions that were taken to block the carnosinase activity during blood collection. Post-hoc analysis revealed that there was a marked difference in plasma carnosinase protein content (p < 0.001) and activity (p = 0.007) between the responders and non-responders (Figure 4A and B). The mean plasma carnosinase protein levels were approximately 2-fold higher in the non-responders (mean ± SD: 91.57 ± 25.37 µg/ml) compared to the responders group (mean ± SD: 44.42 ± 11.22 µg/ml, p < 0.001). Moreover, it seems that there is a clear cut-off value in plasma carnosinase protein content (Figure 4A), as the highest value of the responders was approximately the same as the lowest of the non-responders (55 µg/ml). Likewise, the CN1 activity is approximately 1.5-fold higher in non-responders (mean ± SD: 6.65 ± 1.80 µmol/ml/h) compared to the responders (mean ± SD: 4.53 ± 1.26 µmol/ml/h; p = 0.007, Figure 4B). Also within the responders’ group, the amount of plasma carnosine (AUC) is negatively correlated with the plasma carnosinase protein levels (p = 0.059, r = -0.68, R² = 0.47) and carnosinase activity (p = 0.059, r = -0.73, R² = 0.52). Both women (n = 2) and men (n = 6) and subjects with 5-5 (n = 3), 5-7 (n = 1) and 6-6 (n = 4) CNDP1 genotype were represented in the responders group (Table 1). The mean age of responders (mean ± SD: 21.6 ± 0.7 yrs) was significantly lower (p = 0.045) compared to the mean age of the non-responders (mean ± SD: 23.1 ± 2.6 yrs).

**Urinary carnosine is related to supplementation-induced carnosinemia.**

The urinary carnosine recovery (% of ingested dose) varied largely between 0.23 and 13.27 %. The responders had a significantly (p = 0.006) higher recovery (mean ± SD: 7.7 ± 3.6 %) compared to the non-responders (mean ± SD: 2.9 ± 1.3%, Figure 5A). Furthermore, a strong negative association was observed between the urinary carnosine (% of ingested dose) and the plasma carnosinase activity (p = 0.001, r = -0.64, R² = 0.41, Figure 5B) and protein content (p < 0.001, r = -0.66, R² = 0.44, Figure 5C). There was a trend for a positive correlation between the areas under the curve of
plasma and urinary carnosine within the responders group (n = 8, p = 0.07, r = 0.66, R² = 0.44). The kinetics of urinary carnosine 4h after carnosine supplementation were comparable between the responders and non-responders. The majority was excreted during the first hour (responders: 45.6 %; non-responders: 58.6 %) and thereafter, the concentration slowly began to decline and 90 % - 95 % of the increase in urinary carnosine was disappeared 4h after carnosine ingestion.

**Profiling beta-alanine in plasma after carnosine ingestion**

Figure 6 shows the profile of beta-alanine in plasma after 60 mg/kg carnosine supplementation separated for subjects with or without supplementation-induced carnosinemia. The total amount of beta-alanine detected in plasma (AUC) was significantly higher in the responders group (p = 0.005, + 30%) versus the non-responders. The C<sub>max</sub> was reached after 30 min for non-responders (mean ± SD: 382.6 ± 122.9 µM), after 40 min for responders (mean ± SD: 468.2 ± 66.0µM) and beta-alanine was almost completely removed from the plasma 2h after supplementation. Furthermore, the plasma beta-alanine content was negatively correlated to the plasma carnosinase protein levels (p = 0.001, r = -0.61, R² = 0.37) and activity (p = 0.019, r = -0.47, R² = 0.22).

**Side effects**

During the first hour after supplementation with 60 mg/kg BW carnosine, a total of 6 participants transiently suffered from both headache and paraesthesia symptoms, 2 subjects only from paraesthesia and 2 subjects from headache. The presence of these symptoms was not related to supplementation-induced carnosinemia, CNDP1 genotype or gender. However, subjects complaining about paraesthesia and/or headache had a higher total amount of beta-alanine in urine (symptoms: 93.7 ± 66.8 mg, no symptoms: 48.1 ± 45.6 mg, p = 0.05), although the total amount of plasma beta-alanine was not different compared to subjects without paraesthesia and/or headache (symptoms: 1.4 ± 0.2 mM, no symptoms: 1.3 ± 0.4 mM, p > 0.5).
Discussion

The main finding of this study is that a high carnosinase activity counteracts the presence of circulating carnosine upon an oral challenge. This likely provides a missing link in the pathophysiological mechanism between carnosine, CNDP1 genotype and diabetic complications, as shown in Figure 7. The mechanism of the protective effect of the Mannheim allele in diabetic nephropathy has been attributed to a lower carnosinase activity (16). However, the further link with higher circulating carnosine has never been established. Carnosine has been shown to be a natural scavenger of the hyperglycemia-induced overproduction of reactive oxygen species, AGE’s and reactive aldehydes and is therefore a promising candidate therapeutic molecule in reducing the risk to develop diabetic complications. More interestingly, Riedl et al. (31) recently showed that carnosinase activity is increased by hyperglycemia through N-glycosylation, and is elevated in type-2 diabetic patients. Therefore, hyperglycemia not only directly induces glycoxidative stress, but also indirectly suppresses the endogenous protective mechanism through carnosine, which probably further speeds the development of complications (Figure 7).

The findings of this study implicate that the quantification of the plasma carnosinase protein content could be a reliable tool to determine the risk for developing nephropathy in diabetic patients as there is almost no overlap between subjects with or without supplementation-induced carnosinemia with respect to plasma carnosinase protein content. Interestingly, this indirectly takes the glycemic control into account, as carnosinase protein levels are elevated in hyperglycemic conditions (31). In 2005, Janssen et al. (16) recommended to investigate the potential of the CNDP1 variants in predicting the risk for developing diabetic nephropathy. However, the potential of the CNDP1 gene may be limited as 1) not all patients with homozygosity for the Mannheim allele were protected against diabetic nephropathy (16) and
2) the subjects with supplementation-induced carnosinemia in this study were characterized with both 5-5, 5-7 and 6-6 CNDP1 genotype. Therefore, it would be interesting to investigate whether diabetic patients with a low plasma carnosinase protein content will be more protected against diabetic nephropathy compared to patients with a higher carnosinase protein content (irrespective of CNDP1 genotype).

The carnosinase activity and protein content was shown to be a discriminating factor for supplementation-induced carnosinemia. Consequently, a potential therapeutic strategy to reduce the risk of diabetic nephropathy could be the inactivation of the carnosinase enzyme. In addition to the (CTG)n polymorphism in the CNDP1 gene (10; 16; 24), female gender (6; 10; 29) and increasing age (29) are determinants associated with higher carnosinase activity that can not be manipulated. In vitro experiments, however, revealed that the activity of carnosinase enzyme was markedly decreased in the presence of both homocarnosine (gamma-aminobutyryl-L-histidine) (28; 29) and anserine (beta-alanyl-N1-methylhistidine) (28). Though, the correlation between circulating homocarnosine or anserine levels in fasted subjects and plasma carnosinase activity in vivo is less obvious (28; 29). Importantly, a good glycemic control, either by intervention with insulin, exercise or diet, seems to be crucial for diabetic patients as this not only directly influences oxidative and glycation stress, but also indirectly affects carnosinase activity (31). Besides the inactivation of the carnosinase enzyme, the development of a carnosine-analogue resistant to the carnosinase enzyme would be a promising strategy to prevent patients from diabetic complications. In this light, Aldini et al. (4) recently showed that the enantiomer D-carnosine has the same protective effect as L-carnosine in obese Zucker rats, while it is more resistant to carnosinase activity. However, D-carnosine has a lowered bioavailability as it is less absorbed in respect to L-carnosine and therefore the relevance for human use may be limited (4).
In the assumption that dietary intake of carnosine can diminish the risk for diabetic complications, the lack of carnosine in the diet, as is the case in a vegetarian diet, could be deleterious for the protection against glycation and oxidative stress. Indeed, plasma AGE content of healthy vegetarians has been reported to be higher compared to omnivores (19; 33). Although a low-fat carbohydrate-rich vegetarian diet is often recommended for diabetic patients (for its positive impact on insulin sensitivity, blood pressure, serum lipid profile, etc (21)), the dietary intake of carnosine, perhaps by supplementation, should not be neglected.

Carnosine is, even after meat or carnosine supplementation, hard to detect in human plasma, as a result of the high carnosinase activity. Yet, when several precautions are taken (cooling, use of EDTA tubes and the immediate deproteinization), when pharmacological doses of carnosine (60mg/kg BW) are ingested and when subjects with low carnosinase activity are studied, plasma carnosine levels can be clearly quantified up to 1h after ingestion. In contrast to plasma, urinary carnosine is more easily detectable, even in subjects ingesting their usual diets (0.2µM - 18.6µM) (2; 36). This urinary carnosine excretion is increased after carnosine or meat supplementation with a urinary recovery up to 14% of the ingested amount (12; 38). As there is no increase in urinary carnosine after beta-alanine (2g) and histidine (2g) intake, Gardner et al. (12) hypothesized that the excreted carnosine is not arisen from resynthesis of beta-alanine and histidine after hydrolysis in intestine and/or plasma and that the amount of urinary carnosine is a reflection of plasma carnosine. This hypothesis is confirmed in the present study by the positive correlation between urinary and plasma carnosine in the responders group and by the strong negative relation between urinary carnosine and plasma carnosinase activity and protein content (Figure 5B and 5C). Following carnosine supplementation, urinary carnosine excretion seems to be a reliable and easier measurable estimation of plasma carnosine content, which is equally dependent on serum carnosinase activity and content.
Since renal tubular epithelium is equipped with oligopeptide transporters with a high affinity for carnosine (17; 34), circulating carnosine can be accumulated by the kidney and may provide an additional exogenous source of protective peptides against diabetic metabolites in patients with low levels of carnosinase.

One would expect that beta-alanine, the degradation product of the plasma carnosinase enzyme, would be positively related to the carnosinase activity. However, the opposite is true as the responders group showed a higher total amount of plasma beta-alanine compared to the non-responders. This may suggest that the majority of carnosine is in fact degraded in other compartments and tissues, than the circulation. Thus, low carnosinase activity favors both carnosinemia and beta-alaninemia following carnosine supplementation, although the mechanism for the latter is unclear.

It can be concluded that 1) carnosine can be detected in human plasma following oral ingestion and 2) a high carnosinase activity and content potently counteracts the presence of circulating carnosine. In a diabetic environment, this could impede the ability of carnosine to exert its protective effects against cytotoxic agents leading to diabetic complications. The inhibition of the carnosinase enzyme and the development of a carnosine analogue resistant to carnosinase have to be investigated as potential therapeutic strategies for reducing the risk for diabetic complications.
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Table I

CN1 activity and protein content (mean ± SD) separated for CNDP1 genotype and gender (* p < 0.05 vs. male, † p < 0.1 vs. male). The subjects in the responders group were both males and females and were characterized with both 5-5, 5-7 and 6-6 CNDP1 genotype.

Figure 1

A. HPLC chromatogram of human plasma (subject from the responders group) withdrawn 30 minutes after ingestion of 60mg/kg BW carnosine in a cooled EDTA tube which was immediately deproteinized (solid line) and the same sample spiked with standard carnosine (dotted line). The peak at 15.6 min (carnosine) increased, while all other peaks remained the same (including beta-alanine at 12.1 min).

B. HPLC chromatogram from human plasma (subject from the responders group) withdrawn 40 minutes after ingestion of 60mg/kg BW carnosine in 1/ a cooled EDTA tube which was immediately deproteinized (solid line) and in 2/ a non-cooled heparin tube (not deproteinized before storage, dotted line). The carnosine peak at 15.6 minutes has disappeared and beta-alanine (12.1 min), taurine (13.2 min) and the peak at 14.2 min (partially histidine) were higher in heparin versus EDTA plasma.

Figure 2

The activity of plasma carnosinase enzyme is dependent on the amount of plasma carnosinase protein content as there is a strong positive correlation between CN1 activity and protein content (p = 0.004, r = 0.58, R² = 0.34).

Figure 3

Time course of plasma carnosine of the 8 responders (individual values, mean = solid line) up to 2h after oral administration of 60mg/kg BW carnosine. The increase reached a peak (range: 30.7 – 195.0 µM) at 20 - 60 minutes and rapidly declined within 1-2 hours.
Figure 4
Boxplot, representing the minimum/maximum values, lower/upper quartile and median of CN1 protein content (A) and CN1 activity (B) of the non-responders compared to responders. The plasma carnosinase protein content (A) and activity (B) was significantly lower in the subjects characterized with supplementation-induced carnosinemia after carnosine supplementation (60mg/kg BW) compared to the non-responders group (p < 0.001 and p = 0.007, respectively; ° = outlier).

Figure 5
Urinary carnosine and beta-alanine: A/ The amount of urinary carnosine (black bars, expressed as % of ingested carnosine) is 2.6-fold higher (p = 0.006) in the responders group while there is no difference in urinary beta-alanine (white bars) between the responders and non-responders (respectively 1.6 ± 0.9 vs. 1.4 ± 1.3, p > 0.05). B,C/ Relationship between CN1 activity (B, p = 0.001, r = -0.64, R² = 0.41) / CN1 protein content (C, p < 0.001, r = -0.66, R² = 0.44) and urinary carnosine (% of ingested dose) during the 4h after carnosine supplementation (60mg/kg BW).

Figure 6
The mean (± SD) area under the curve of plasma beta-alanine (µM) is significantly higher in the responders (dotted line) versus the non-responders group (solid line, p = 0.005, +30%).

Figure 7
Proposed pathophysiological mechanism linking carnosine and CNDP1 genotype to diabetic complications. * denotes effects that are now supported by the findings of the current study in humans.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>CN1 activity ($\mu$mol/ml/h)</th>
<th>CN1 protein ($\mu$g/ml)</th>
<th>N° of responders within subgroup (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNDP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-5</td>
<td>5.6 ± 1.7</td>
<td>82.3 ± 32.3</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>6-6/5-7</td>
<td>6.3 ± 2.2</td>
<td>119.7 ± 31.3</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5.3 ± 1.9</td>
<td>67.3 ± 26.0</td>
<td>6/15 (40%)</td>
</tr>
<tr>
<td>Female</td>
<td>6.8 ± 1.6 *</td>
<td>92.5 ± 32.6 †</td>
<td>2/10 (20%)</td>
</tr>
</tbody>
</table>