



(51) International Patent Classification:

A61K 38/05 (2006.01) A23L 33/18 (2016.01)

A23L 33/17 (2016.01)

EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(21) International Application Number:

PCT/EP2017/084075

(22) International Filing Date:

21 December 2017 (21.12.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

16206052.9 22 December 2016 (22.12.2016) EP

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

(71) Applicants: UNIVERSITEIT GENT [BE/BE]; Sint-Pietersnieuwstraat 25, 9000 Gent (BE). UNIVERSITÀ DEGLI STUDI DI MILANO [IT/IT]; Via Festa del Perdono 7, 20122 Milano (IT).

(72) Inventors: DERAVER, Wim; Petrus De Broestraat 18, 9030 Mariakerke (BE). EVERAERT, Inge; Koolstraat 24, 9290 Berlare (BE). BLANCQUAERT, Laura; Lostraat 79, 9920 Lovendegem (BE). GIANCARLO, Aldini; Via Ambrosini 5, 20900 Monza (IT). REGAZZONI, Luca, Giovanni; Via Padri Serviti 21, 23801 Calolziocorte (LC) (IT). BARON, Giovanna; Via Primo Maggio 31, 21046 Malnate (VA) (IT).

(74) Common Representative: UNIVERSITEIT GENT; TechTransfer Office - iGent Tower Technologiepark-Zwijnaarde 15 (3rd floor), 9052 Gent-Zwijnaarde (BE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

(54) Title: MIXTURE OF DIPEPTIDES TO IMPROVE EXERCISE PERFORMANCE

(57) Abstract: The present invention relates to the field of performance-enhancing sport supplements. More specifically, the present invention discloses that oral administration of a mixture of the dipeptides carnosine and anserine to a subject once before the start of an exercise significantly improves the performance of said exercise. Moreover, the invention discloses that the latter ergogenic effect is due to the capability of said dipeptides to scavenge the reactive aldehyde acrolein. Hence, the invention relates to an easy-to-use supplement that quickly removes reactive products involved in muscle fatigue which results in an increase in power production during high-intensity exercises.

Mixture of dipeptides to improve exercise performance

Technical field of the invention

The present invention relates to the field of performance-enhancing sport nutritional supplements.

5 More specifically, the present invention discloses that oral administration of a mixture of the dipeptides carnosine and anserine to a subject once before the start of an exercise significantly improves the performance of said exercise. Moreover, the invention discloses that co-ingestion of carnosine markedly improves the in vivo bio-availability of a dose of ingested anserine, leading to a synergistic effect of carnosine and anserine ingestion on circulating dipeptide levels. Finally, the
10 invention discloses that the latter ergogenic effect likely due to the capability of said dipeptides to scavenge the reactive aldehyde acrolein. Hence, the invention relates to an easy-to-use supplement that quickly removes reactive products that may compromise muscle function, which results in an increase in power production during high-intensity exercises.

Background art

15 Carnosine and anserine are two histidine-containing dipeptides (HCD) which are present in high concentrations in skeletal muscle of mammals and are therefore abundantly present in the daily food of non-vegetarian persons. Beta-alanine and histidine are the two constituent amino acids of carnosine, while anserine (beta-Alanyl-N(pi)-methyl-L-histidine) is the methylated variant of carnosine. The long-term administration of beta-alanine has been shown to be an effective method to increase
20 muscle carnosine levels. Moreover, this disturbance of the muscle carnosine homeostasis is associated with enhanced high-intensity exercise performance (Hill et al. 2007), especially for exercises lasting between 1 and 6 min (Hobson et al. 2012).

As this long-term beta-alanine supplementation protocol is, although effective, quite inefficient, it was tested whether the acute pre-exercise ingestion of carnosine is a good alternative for exercise
25 enhancement. Specially, it was aimed to test whether an elevation of circulating carnosine levels is as effective as enhancing muscle carnosine levels in the light of exercise enhancement. Unfortunately, the bioavailability of carnosine in the circulation following supplementation is very low in humans. This is due to the fast breakdown of carnosine in the circulation by the very active human carnosinase-1 enzyme. Everaert et al. (2012) showed for example that a high dose of 60 mg/kg BW pure carnosine
30 was needed to induce a short elevation of plasma carnosine following carnosine supplementation in some, but even not in all subjects (7 out of 23). Interestingly, this carnosinemia (i.e. the presence of

carnosine in the circulation) was only present in the subjects with the lowest serum carnosinase activity. Moreover, this pharmacological dose elicited some side-effects, like headache and paresthesia, in 1/3rd of the subjects, and can therefore not be used as pre-exercise supplement. Therefore, Baguet et al. (2014) tested whether the acute ingestion of a lower dose of carnosine (20 mg/kg BW) could elicit improvements in exercise performance. However, the carnosinemia was not detectable in most of the subjects and the performance of high-intensity cycling was not affected. Hence, it is clear that oral and acute administration of the dipeptide carnosine to a subject once before the start of an exercise -in a dose that does not elicit side-effects- does not improve the performance of said exercise! There is thus a need to find an alternative and efficient, performance-enhancing supplement.

In an alternative attempt, the methylated variant of carnosine, anserine, which is likely similarly bio-active, was explored. In vitro experiments revealed that anserine is much less prone to hydrolysis by carnosinase (Pegova et al. 2000, Yeum et al. 2010, Peters et al. 2011). By extracting anserine from tuna, Kubomura et al. (2009) showed that anserine (2g/60kg BW) was (at least partly) absorbed intactly and hydrolyzed to its two constituent amino acids within 2-4h in the blood circulation of humans. Furthermore, report about the effect of acute pre-exercise ingestion of extracted anserine on exercise performance in humans demonstrated that the administration of 0.4 or 2g anserine, one hour before Wingate-type exercise test, was not effective in improving Wingate performance (Marine Active, YSK company information, 2013, EP 1210940 B1 and US2009087495A1). Hence, it is doubtful whether oral and acute administration of the dipeptide anserine to a human subject once before the start of an exercise is capable of improving the performance of said exercise. There is thus again a need to find an alternative and efficient, performance-enhancing supplement.

In South-East Asia, the use of chicken extracts, which contain carnosine: anserine (1:2–1:3 ratio, respectively), but which are also rich in other bioactive compounds, such as proteins, free amino acids, taurine, ... and several minerals and vitamins (Li et al. 2012), is very popular. It is said to improve mental health, immune function, hypertension, anxiety, These chicken extracts have also been tested for their ergogenic (= performance enhancing) potential. However, the results are so far inconclusive: one study showed that the pre-exercise intake of the chicken breast extract (denominated as CBEXTM) enhanced the power output of 2 sets of repeated sprints (Suzuki et al. 2004), while it was not able to enhance performance in a single set of similar repeated sprints (Suzuki et al. 2006). Moreover, it cannot be excluded that other compounds present in said chicken extract such as taurine, vitamins, minerals, ... might be responsible for the potential bioactive effect of the extract. Hence, it is unclear whether oral administration of an extract comprising –among other bioactive compounds- the dipeptides carnosine and anserine to a subject once before the start of an exercise is capable to improve the

performance of said exercise. There is thus again a need to find an alternative and efficient, performance-enhancing supplement.

Baba et al. (2013) demonstrated that endogenous carnosine reacts in vivo with the highly reactive aldehyde acrolein, which results in urinary excretion of both the oxidized (carnosine-propanal) and reduced (carnosine-propanol) form of carnosine-acrolein adducts. Regazzoni et al. (2016) further showed that urinary excretion of carnosine-acrolein adducts is increased in some, but not all, overweight subjects who received carnosine (2g/day) for 12 weeks. *In vitro* studies showed that also anserine is able to quench reactive aldehydes including HNE (Aldini et al. 2005) and acrolein (Baba et al. 2013). However, it is again completely unknown whether oral administration of a mixture comprising the dipeptides carnosine and anserine to a subject once before the start of an exercise is capable to improve –via the acrolein quenching capabilities of both dipeptides- the performance of said exercise.

Brief description of figures

Figure 1 (experiment 1): Bioavailability of chemically synthesized L-anserine. (A) Mean and individual (dashed line) profiles of plasma anserine (μM) following acute ingestion of 20 mg/kg BW chemically synthesized anserine. Plasma anserine was increased following ingestion in all subjects (7/7). (B) The amount of anserinemia (AUC over 3h) is correlated to the serum carnosinase activity ($R^2 = 0.72$).

Figure 2 (experiment 2): (A) The degradation of anserine in isolated human plasma is much slower compared to carnosine (half-life 7.8 vs. 1.7 minutes). (B) The degradation of anserine is even more counteracted when carnosine is added to the medium. The anserine-stabilizing effect of carnosine was independent of carnosinase activity.

Figure 3 (experiment 3): (A) Profile of plasma anserine (μM , mean $\pm$ SD) upon acute supplementation with 25 mg/kg BW anserine (dashed line) or 25 mg/kg BW anserine + 25 mg/kg BW carnosine (solid line). The increase in plasma anserine is markedly enhanced (2.6 – fold higher AUC) when anserine is co-administered with carnosine compared to anserine alone ($p \leq 0.1$). (B) Peak plasma concentration of anserine is also 2.6-fold higher ($p \leq 0.05$) following administration with carnosine and anserine versus anserine alone.

Figure 4 (experiment 4): Plasma histidine-containing dipeptides 45 minutes following ingestion of placebo, carnosine (25 mg/kg BW), anserine (25 mg/kg BW), or mixture of carnosine + anserine (each 25 mg/kg BW). Plasma histidine-containing dipeptides were absent in placebo and carnosine condition, while it was significantly increased in anserine and carnosine/anserine condition (* $p \leq 0.05$) versus

placebo. The sum of histidine-containing dipeptides is 2.1-fold higher when a mixture of carnosine and anserine was ingested versus anserine alone ($p \leq 0.1$).

Figure 5 (experiment 5): (A) Mean power output (W/kg BW) was significantly improved in the first 5s of a 30s all-out cycling performance in the Carnosine/Anserine supplementation vs. placebo condition; * $p < 0.05$ vs. placebo. (B) Individual ratio's (between Carnosine/Anserine and placebo condition) in mean power during the 1st 5 seconds of the Wingate test. Subjects performed on average 6 ± 11 % better when carnosine + anserine was supplemented.

Figure 6 (experiment 6): (A) Difference in peak power output (W/kg BW) between the pre-supplementation performance and the post-supplementation performance (60 minutes after ingestion) for the 5 x 6s all-out repeated sprints. Cycling performance was significantly more improved in the 30 mg/kg BW Carnosine/Anserine supplementation vs. placebo condition, while this was not the case for 10 mg/kg BW and 20 mg/kg BW; * $p < 0.05$ vs. placebo. (B) Difference in peak torque (N m/kg BW) between the pre-supplementation performance and the post-supplementation performance (60 minutes after ingestion) for the 3 x 3s isometric MVC. Peak torque was significantly less declined/more improved in the 30 mg/kg BW Carnosine/Anserine supplementation vs. placebo condition; * $p < 0.05$ vs. placebo; $\$ p < 0.1$ vs. placebo.

Figure 7 (experiment 5): (A) The effect of high-intensity cycling (6-minutes high-intensity + 30s all-out cycling) and supplementation with 20 mg/kg BW carnosine and 20 mg/kg BW anserine on urinary carnosine – and anserine-acrolein adducts (sum of oxidized and reduced form); * $p < 0.05$ vs. pre ; # $p < 0.05$ between dipeptides. (B) Correlation between difference in urinary anserine-acrolein adducts and difference in mean power over 30s Wingate in Carnosine/Anserine versus placebo condition.

Summary of invention

The present invention relates to the field of performance-enhancing sport supplements. More specifically, the present invention discloses that oral administration of a mixture essentially consisting of the dipeptides carnosine and anserine to a subject once before the start of an exercise significantly improves the performance of said exercise. More in particular, the present invention discloses a mixture comprising carnosine and anserine wherein the relative weight of carnosine, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80%. More specifically, the present invention discloses a mixture comprising carnosine and anserine wherein the relative

weight of carnosine, when compared to the total weight of carnosine and anserine in said mixture, is about 50%.

In other words, the present invention relates to a mixture consisting essentially of carnosine and anserine for use to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise. In other words, the present invention relates to the usage of a mixture as indicated above to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise.

More specifically, the present invention relates to a mixture as indicated above, wherein said single dose of said mixture is administered 3 hours or less before the start of said exercise. In other words, the present invention relates to the usage of a mixture as indicated above, wherein said single dose of said mixture is administered 3 hours or less before the start of said exercise.

More specifically, the present invention relates to a mixture as indicated above, wherein said single dose of said mixture is administered 1 hour or less before the start of said exercise. In other words, the present invention relates to the usage of a mixture as indicated above, wherein said single dose of said mixture is administered 1 hour or less before the start of said exercise.

More specifically, the present invention relates to a mixture as indicated above, wherein said single dose of said mixture is administered about 40 minutes before the start of said exercise. In other words, the present invention relates to the usage of a mixture as indicated above, wherein said single dose of said mixture is administered about 40 minutes before the start of said exercise.

The present invention further relates to the usage of a mixture as indicated above, wherein the relative weight of carnosine in said mixture, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80 %.

Moreover, the present invention relates to the usage of a mixture as indicated above, wherein the relative weight of carnosine is about 50%. In other words, the present invention relates to a mixture as indicated above, wherein the anserine/carnosine mixture is administered in a ratio 1:1.

The present invention further relates to the usage of a mixture as indicated above, wherein said dose is more than 20mg/kg body weight of carnosine and anserine combined.

Furthermore, the present invention relates to the usage of a mixture as indicated above, wherein said dose is at least 40mg/kg body weight of carnosine and anserine combined.

The present invention also relates to the usage of a mixture as indicated above wherein said mixture is administered as a capsule.

The present invention further relates to the usage of a mixture as indicated above wherein said mammal is a human.

- 5 The present invention further relates to the usage of a mixture as indicated above wherein said human is an athlete and wherein said exercise is a high-intensity exercise.

Description of invention

The present invention discloses a mixture comprising carnosine and anserine wherein the relative weight of carnosine, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80%. More specifically, the present invention discloses a mixture comprising carnosine and anserine wherein the relative weight of carnosine, when compared to the total weight of carnosine and anserine in said mixture, is about 50%. The present invention relates to the usage of a mixture as indicated above to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise.

- 15 More specifically, the present invention relates to mixture as indicated above consisting essentially of carnosine and anserine for use to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise.

Carnosine and anserine as effective components of the mixture of the present invention are known substances and are commercially available. Beta-alanine and histidine are the two constituent amino acids of carnosine, while anserine (beta-Alanyl-N(pi)-methyl-L-histidine) is the methylated variant of carnosine. Carnosine and anserine can be prepared by chemical synthesis, by fermentation or by extracting both compounds from natural products such as chicken and fish meat as is -for example- described in US2009/0087495.

- 25 The term 'comprising' indicates that said mixture may include –besides anserine and carnosine- other compounds.

The term 'consisting essentially of' indicates that said mixture may include –besides anserine and carnosine- other compounds provided they do not negatively affect the effect (i.e. improving the performance of an exercise) of said mixture. Non-limiting examples of compounds that do not negatively affect the effect of said mixture are physiologically permitted compounds necessary for formulation such as additives, carriers, excipients, diluents and the like. The term 'consisting essentially of' also includes embodiments of mixtures which 'consist of' carnosine and anserine.

In another embodiment, the present invention also relates to mixture as indicated above consisting of carnosine and anserine for use to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise.

The terms 'improve an exercise performance' relates to the fact that the present invention demonstrates that acute pre-exercise co-ingestion of carnosine and anserine significantly ($P < 0.05$) improves power output (which is a non-limiting example of an 'exercise performance') during high-intensity cycling (which is a non-limiting example of an 'exercise') when compared to the power output generated under control conditions. Hence, the term 'improve an exercise performance' relates to any activity or component of an exercise task which becomes significantly improved when an acute pre-exercise co-ingestion of carnosine and anserine occurs and when compared to the performance under control conditions. An exercise can be any exercise but is preferably a high-intensity exercise such as high intensity cycling, high intensity running, high intensity efforts interspersed by short recovery intervals during team sports such as soccer, basketball, rugby and the like.

The terms 'wherein a single dose of said mixture is orally administered once before the start of said exercise' relate to the timing and mode of administration of the mixture of the present invention and has the same meaning as "an acute pre-exercise co-ingestion of carnosine and anserine". In other words, a mixture as indicated above comprising or consisting essentially of anserine and carnosine is administered once or one single time (i.e. 'acute' administration in contrast to 'chronic' administration) through the mouth of a subject and this administration is undertaken some time period before beginning the exercise which performance is wished to be improved.

More specifically, the present invention relates to a mixture as indicated above, wherein said single dose of said mixture is administered 3 hours or less before the start of said exercise. If -for example- a subject is planning to begin his/her exercise at 4:00 pm, the latter dose is for -example- preferably administered at 1:00, 1:30, 2:00, 2:30, 3:00, 3:30 pm or at any time between 1:00 pm and about 3:30 pm.

Even more specifically, the present invention relates to a mixture as indicated above, wherein said single dose of said mixture is administered 1 hour or less before the start of said exercise. If -for example- a subject is planning to begin his/her exercise at 4:00 pm, the latter dose is for -example- preferably administered at 3:00, 3:15, 3:30 pm or at any time between 3:00 pm and about 3:30 pm.

More specifically, the present invention relates to a mixture as indicated above, wherein single dose of said mixture is administered about 40 minutes before the start of said exercise. If -for example- a subject is planning to begin his/her exercise at 4:00 pm, the latter dose is for -example- preferably administered at 3:10, 3:15, 3:20, 3:25, 3:30 pm or at any time between 3:10 pm and about 3:30 pm.

The present invention relates to a mixture as indicated above, wherein the relative weight of carnosine in said mixture, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80 %. The latter terms indicate that if –for example- the total weight of carnosine and anserine in said mixture is 3000 mg that the latter mixture can -for example- contain 1200 mg of carnosine and 1800 mg of anserine, or, 1350 mg of carnosine and 1650 mg of anserine, or, 1500 mg of carnosine and 1500 mg of anserine, or, 1650 mg of carnosine and 1350 mg of anserine, or, 1800 mg of carnosine and 1200 mg of anserine, or 1950 mg of carnosine and 1050 mg of anserine, or, 2100 mg of carnosine and 900 mg of anserine, or, 2250 mg of carnosine and 750 mg of anserine, or, 2400 mg of carnosine and 600 mg of anserine, or any other (relative) weight of carnosine between 1200 mg and 2400 mg.

In a preferred embodiment, the present invention relates to a mixture as indicated above, wherein the relative weight of carnosine is about 50%. In other words, the present invention relates to a mixture as indicated above, wherein the anserine/carnosine mixture is administered in a weight ratio 1:1.

The present invention further relates to a mixture as indicated above, wherein said dose is more than 20mg/kg body weight of carnosine and anserine combined. A dose of more than 20mg/kg body weight of carnosine and anserine combined means a dose of 21, 22, 23, 24 , 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, ... mg/kg body weight. A none limiting example of the latter dose is a dose of 39 mg/kg body weight wherein 15,6 mg/kg BW carnosine is present and 23,4 mg/kg BW anserine is present, or wherein 19,5 mg carnosine is present and wherein 19,5 mg/kg BW of anserine is present.

The present invention relates to a mixture as indicated above, wherein said dose is at least 40mg/kg body weight of carnosine and anserine combined.

A dose of at least 40mg/kg body weight of both carnosine and anserine means a dose of 40, 60, 80, 85, 90, 95 mg/kg body weight or any dose of carnosine and anserine combined between 40 and 95 mg/kg body weight. A non-limiting example of the latter dose is a dose of 40 mg/kg body weight of carnosine and anserine combined wherein 16 mg/kg BW carnosine is present and wherein 24 mg/kg BW anserine is present or wherein 22 mg/kg BW carnosine is present and wherein 18 mg/kg BW anserine is present. Preferred doses of the present invention is a dose of 40 mg/kg BW of carnosine and anserine combined wherein 20 mg/kg BW carnosine is present and wherein 20 mg/kg BW anserine is present or a dose of 60 mg/kg BW of carnosine and anserine combined wherein 30 mg/kg BW carnosine is present and wherein 30 mg/kg BW anserine is present.

As mentioned above, the mixture of the present invention may contain, in addition to anserine and carnosine, compounds that do not negatively affect the effect of said mixture such as physiologically permitted compounds necessary for formulation such as additives, carriers, excipients, diluents and

the like. The mixture of the present invention can thus be formulated as a tablet, powder, granule, capsule and the like. In another embodiment of the present invention, the mixture of the present invention can be added to a sport food matrix such as an energy drink or an energy bar. In a preferred embodiment, the present invention relates to a mixture as indicated above wherein said mixture is administered as a capsule.

The present invention relates to improving an exercise performance by a mammal. The latter mammal can be any mammal of which an increased exercise performance is desired such as a horse, a dog, a human being... In a preferred embodiment, the present invention relates to a mixture as indicated above wherein said mixture is administered to a human. The present invention further relates to a mixture as indicated above wherein said human is an athlete and wherein said exercise is a high-intensity exercise such as high intensity cycling, high intensity running, high intensity efforts interspersed by short recovery intervals during team sports such as soccer, basketball, rugby and the like.

The present invention further relates to a process to improve an exercise performance by a mammal, comprising orally administering once before the start of said exercise an effective amount of a mixture comprising or consisting essentially of carnosine and anserine to a mammal in need thereof. The latter 'before the start of said exercise' is 3 hours or less before the start of said exercise, 1 hour or less before the start of said exercise, or, about 40 minutes before the start of said exercise as indicated above. The terms 'an effective amount' refer to 'an amount wherein the relative weight of carnosine in said mixture, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80 %, or, is about 50%' as indicated above. More specifically, the terms 'an effective amount' refer to 'a dose of more than 20mg/kg body weight of carnosine and anserine combined ', or, 'a dose of at least 40mg/kg body weight of carnosine and anserine combined' as indicated above. The latter mixture is preferably administered as a capsule in a mammal which is preferably a human, and more preferably an athlete performing a high-intensity exercise as indicated above.

The present invention will further be illustrated by the following, non-limiting examples.

Examples

Methods

Subjects gave their informed consent for all described experiments and the studies were approved by the Local Ethics Committee (Ghent University Hospital).

Bioavailability of chemically synthesized anserine

Experiment 1: To examine the bioavailability, 7 young healthy subjects were orally supplemented with 20 mg/kg BW chemically synthesized L-anserine. After an overnight fast (at least 8h), an indwelling catheter was inserted in an antecubital vein and blood was withdrawn before and 20, 40, 60, 80, 120, 180 minutes following oral supplementation of anserine (dissolved in 250ml water). Blood samples for determination of anserine were collected in pre-cooled (4°C) EDTA plasma tubes. Plasma samples were deproteinized with SSA (35%) and stored immediately at -20°C until further analysis for anserine content with LC-MS² (LOD 0.2µM). The subjects were allowed to drink water ad libitum. Plasma carnosinase activity was quantified by measuring the liberated amount of histidine (OPA derivatisation) fluorometrically following carnosine addition to plasma.

In vitro hydrolysis of carnosine and anserine

Experiment 2: L-Anserine and L-carnosine, either alone or in combination, were added to human heparin plasma in a concentration of 100µM of each dipeptide. The degradation of anserine and carnosine by the endogenous serum carnosinase enzyme was stopped on different time points (0, 1, 2, 3, 5, 7, 10, 15, 25, 40 min) by adding 35% of sulfosalicylic acid (9:1) and removing the proteins by centrifugation. The remaining amount of histidine-containing dipeptides was measured fluoremetrically by HPLC (AccQTag method).

In vivo bio-availability of oral ingestion of anserine with or without co-ingestion of carnosine

Experiment 3: Five young healthy subjects were on two separate occasions (at least one week in between) orally supplemented with either 25 mg/kg BW L-anserine or 25 mg/kg BW L-anserine + 25 mg/kg BW L-carnosine (capsules). After an overnight fast (at least 8h), subjects received a standardized breakfast, free of carnosine and anserine, and 1h15min later they were supplemented with either 25 mg/kg BW anserine or 25 mg/kg BW anserine + 25 mg/kg BW carnosine in capsules. Blood was withdrawn from an antecubital vein before and 20, 40, 60, 80 and 100 minutes following ingestion of the supplements.

Experiment 4: On a separate occasion, fifteen healthy subjects, were on 4 separate occasions (at least one week in between) orally supplemented with (A) maltodextrine as placebo, (B) 25 mg/kg BW carnosine, (C) 25 mg/kg BW anserine or (D) 25 mg/kg BW carnosine + 25 mg/kg BW anserine. After an overnight fast (at least 8h), subjects received a standardized breakfast, free of carnosine and anserine, followed (1h45min later) by the ingestion of the supplements in capsules. Blood was withdrawn from an antecubital vein before and 45 minutes after ingestion. Blood samples, for determination of anserine and carnosine, were collected in pre-cooled (4°C) EDTA plasma tubes. Plasma samples were deproteinized with SSA (35%) and stored immediately at -20°C until further analysis with HPLC (AccQTag method).

Exercise performance and mechanism of action

Experiment 5: Eighteen trained men volunteered to participate in this double-blind, placebo-controlled, crossover study. During the first visit of the participants, a graded exercise cycling test until exhaustion was performed to determine the power output equivalent to 50% of the difference between ventilatory threshold and $\text{VO}_{2\text{peak}}$ (50% Δ). On the days of experiments (at least one week between the two conditions), subjects were asked 1/ to consume no meat or fish the evening before the testday, 2/ to refrain from exercise training 24h before the test day, 3/ and to arrive in a fasted condition. At arrival, subjects received a standardized breakfast, free of carnosine and anserine, and 1h15min later they were supplemented with either 20 mg/kg BW anserine and 20 mg/kg BW carnosine in capsules or a similar amount of maltodextrine capsules. Thirty-five minutes following supplementation, subjects started a standardized warm-up for 5 minutes. In order to fatigue the subjects, a 6-minutes high-intensity cycling test at a power output equivalent to 50% of the difference between ventilatory threshold and $\text{VO}_{2\text{peak}}$ was performed immediately after the warm-up. Following 6 minutes of active rest, a 30s all-out Wingate test was performed to evaluate the recovery capacity of the subjects. Capillary blood samples were collected before and after both exercises and analyzed with Radiometer ABL90 series for pH, HCO_3^- , lactate and several electrolytes (Na^+ , K^+ , Ca^{2+} , Cl^-). A subgroup of 7 subjects collected their urine for 24h following the supplementation and the other 11 out of 18 subjects acutely collected urine pre and post (1h20min) exercise, which was analysed for carnosine – and anserine – acrolein adducts with LC-MS² and corrected for urinary creatinine content.

Dose-response relationship of dipeptide mixture ingestion on exercise performance

Experiment 6: In a second performance experiment, the dose-response relationship was determined for ergogenic effects of the dipeptide mixture. Eleven trained men volunteered to participate in this double-blind, placebo-controlled, crossover study. During the first visit, participants were familiarized with the different performance exercises. The first performance parameter consisted of 3 maximal isometric voluntary contractions (MVC) of the knee extensors with the knee flexed at 90°. Contraction was sustained for 3s and repetitions were separated by 20s rest intervals. Secondly, subjects performed a repeated sprint ability (RSA) test consisting of 5 x 6s all-out cycle sprints every 30s. During the 24s recovery between sprints, subjects were allowed to cycle at a low cadence (below 70rpm). At arrival, subjects received a standardized breakfast free of carnosine and anserine. A standardized cycle warm-up (5min cycling at 80W) was started 1h5min after breakfast. Thereupon, both exercises were performed for the first time (3x3s isometric MVC and 5x6s repeated sprints). 1h30min after they consumed their breakfast, subjects were supplemented with either 10 mg/kg BW, 20 mg/kg BW or 30 mg/kg BW of each L-carnosine and L-anserine in capsules (same amount of carnosine and anserine for each supplementation condition, for example 10mg/kg BW carnosine and 10mg/kg BW anserine) or a

similar amount of maltodextrine capsules (placebo). Fifty minutes following supplementation, subjects again performed the standardized warm-up for 5 minutes and executed the performance exercise tests. Both mean power, peak power and total power delivered was evaluated for both exercise performances.

5 Results

Chemically synthesized anserine is well absorbed and bioavailable in all tested subjects

None of the subjects of experiment 1 reported side-effects following the acute administration of the chemically synthesized anserine. Interestingly, all subjects had a measurable increase in plasma anserine following ingestion of the nutritionally relevant dose of 20mg/kg BW anserine. 10 Supplementation of 20mg/kg BW anserine resulted in a mean ($\pm$ SD) maximum increase of $3.12 \pm 2.52\mu\text{M}$ (range: 0.38 – 6.81 μM) at on average 20-40 minutes after supplementation, which rapidly declined within 1-3 hours (Figure 1a). Remark that even 60 minutes following anserine administration all subjects had still elevated plasma anserine levels. Interestingly, both carnosinase content ($R^2 = 0.66$, data not shown) and carnosinase activity ($R^2 = 0.72$, Figure 1b) are inversely correlated with the 15 magnitude of anserinemia (expressed as AUC) following 20 mg/kg BW anserine ingestion.

Carnosine slows anserine degradation in human plasma in vitro and in vivo

Experiment 2 (In vitro): The in vitro analysis of degradation of carnosine and anserine in isolated human plasma confirmed that L-carnosine is much more prone to hydrolysis by the serum carnosinase enzyme 20 in comparison with L-anserine (Figure 2a). The half-life of 100 μM carnosine is 1.7 min versus 7.8 min for anserine. The degradation of L-anserine is even more attenuated in the presence of carnosine, a competitive substrate for serum carnosinase. Adding carnosine (100 μM) together with anserine (100 μM) to the plasma, results in an improvement of approximately 2 minutes in half-life of anserine in comparison with only anserine (100 μM , Figure 2b). The stabilization effect of carnosine towards 25 anserine degradation occurred in all 4 tested samples, independently of the plasma carnosinase activity (range: 0.2- 2.3 $\mu\text{mol/ml/h}$).

Experiment 3 (In vivo): In the first in vivo test comparing anserine versus anserine+carnosine in five subjects, none of the subjects reported side-effects following the acute ingestion of anserine (25 mg/kg BW) or combination of carnosine and anserine (both 25 mg/kg BW). All except one subjects showed 30 clear anserinemia from 40 – 100 minutes following supplementation in both conditions. However, the increase in plasma anserine was much more prominent (2.6-fold increase in AUC, $p \leq 0.1$) when anserine was co-ingested with carnosine (Figure 3a). Plasma anserine reached a peak concentration of $61.8 \pm 44.5 \mu\text{M}$ following anserine and $105.4 \pm 91.9 \mu\text{M}$ following co-administration of anserine with

carnosine ($p \leq 0.05$, Figure 3b). Carnosinemia ($C_{\max}$: $58.8 \pm 41.0 \mu\text{M}$) was observed in 3 out of 5 subjects at 40 – 80 minutes following carnosine and anserine ingestion and, logically, not following anserine ingestion. Taking total plasma histidine-containing dipeptides into account, a pronounced 3.3-fold increase in AUC and peak concentration ($p \leq 0.5$) was observed when carnosine was co-administered with anserine versus anserine alone.

Experiment 4 (in vivo): The second in vivo test compared placebo vs. carnosine (25 mg/kg BW) vs. anserine (25 mg/kg BW) vs. anserine+carnosine (both 25 mg/kg BW) in fifteen subjects. None of subjects reported side-effects following these acute supplementation protocols. No carnosine or anserine could be detected in human plasma before any supplementation. The histidine-containing dipeptide levels were not enhanced following supplementation with either placebo or carnosine. A marked anserinemia (mean $\pm$ SD: $23.2 \pm 24.7 \mu\text{M}$) was observed in 10/15 subjects following anserine supplementation. Ingesting a mixture of carnosine and anserine resulted in higher anserinemia (mean $\pm$ SD: $42.5 \pm 53.4 \mu\text{M}$, $p \leq 0.1$ vs. anserine alone) in 11/15 subjects and carnosinemia (mean $\pm$ SD: $53.0 \pm 9.8 \mu\text{M}$) in 2/15 subjects. The total sum of circulating histidine-containing dipeptides was 2.1-fold higher when a mixture of carnosine and anserine was ingested versus anserine ingestion in isolation, and even more so compared to carnosine ingestion in isolation, which did not result in any plasma histidine-containing dipeptide profile (Figure 4).

As carnosine slows down the anserine degradation in human plasma, the supplementation of an equal dose of carnosine (10-20-30 mg/kg BW) and anserine (10-20-30 mg/kg BW) was used in further performance experiments.

Pre-exercise ingestion of carnosine/anserine enhances high-intensity exercise performance

Experiment 5: A Wingate test is a maximal cycling exercise test performed during 30 seconds. The highest power is generated during the first 5 seconds. The average power that was produced during the first 5 seconds of that Wingate test was significantly improved after prior ingestion of the Carnosine/Anserine mixture ($p = 0.04$, Figure 5a). Subjects performed on average $6 \pm 11\%$ better during the first 5 seconds of a Wingate test due to the supplementation of Carnosine/Anserine (Figure 5b).

Experiment 6: In the dose-response relationship study in 11 subjects, all subjects performed better during the second set of sprints compared to the pre-supplementation sprints (subjects performed on average $2.6 \pm 1.9\%$ better during in the placebo condition). However, the peak power that was produced during the 5 x 6s all-out sprints was significantly more improved after prior ingestion of the Carnosine/Anserine mixture in a dose of 30 mg/kg BW ($p = 0.013$ vs. placebo, Figure 6a), while this was not the case for the 10 mg/kg BW and 20 mg/kg BW Carnosine/Anserine conditions. In contrast, the peak torque that was produced during the 3 x 3 s isometric MVC's was decreased in the post-

supplementation test compared to pre-supplementation in the placebo condition ($-4.41 \pm 11.2\%$), but this tended to be counteracted by 20 mg/kg BW Carnosine/Anserine supplementation ($p < 0.1$ vs. placebo, (Figure 6b) and was significantly reversed by 30 mg/kg BW Carnosine/Anserine supplementation ($p < 0.05$ vs. placebo, Figure 6b).

5 ***Novel link between acrolein quenching and exercise performance***

In experiment 5, the evolution of pH, lactate or bicarbonate during both the 6' cycling test and the all-out Wingate test was not affected by supplementation. Similarly, also the amount of electrolytes (Na^+ , K^+ , Ca^{2+} , Cl^-) was not affected by the administration of carnosine and anserine.

As the mechanism of action could not be explained by differential changes in blood gases or electrolytes, it was tested whether the reactive carbonyl stress was affected by administration of carnosine and anserine. Exercise effectively increased the production of the reactive aldehyde acrolein and its adducts, as the total carnosine-acrolein adduct content in urine of non-supplemented subjects was enhanced compared to pre-exercise (*, $p = 0.05$, Figure 7a). In contrast to carnosine, the urinary excretion of anserine-acrolein adducts was not enhanced following exercise in the placebo condition. However, when subjects were acutely supplemented with the Carnosine/Anserine mixture, the anserine-acrolein adducts significantly increased 1h20min post-supplementation ($p < 0.05$ vs. placebo). The total anserine-acrolein adducts production was significantly (#, $p < 0.05$) and remarkably (17-fold) higher compared to the formation of carnosine-acrolein adducts (1-fold, Figure 7a). It can therefore be stated that exogenous anserine is a much better acrolein-quencher in comparison to exogenous carnosine.

Interestingly, the quenching/elimination of acrolein-adducts during fatiguing exercise is possibly involved in the performance-enhancing effect of the dipeptide ingestion. The subjects with the highest difference in urinary excretion of anserine-acrolein adducts between the 2 conditions had a higher mean power over 30s when supplemented with Carnosine/Anserine versus the placebo condition ($r = 0.75$, $R^2 = 0.56$, $p = 0.05$; Figure 7b). This correlation could not be confirmed for urinary carnosine-acrolein adducts ($p > 0.1$), further indicating that anserine is the more important and functionally relevant acrolein quencher in humans in vivo. In summary, the more acrolein was quenched by anserine, the more power that could be delivered during 30s all-out Wingate test.

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Claims

1. A mixture comprising carnosine and anserine wherein the relative weight of carnosine, when compared to the total weight of carnosine and anserine in said mixture, ranges from 40 to 80%.
2. A mixture according to claim 1, wherein the relative weight of carnosine is about 50%.
3. Use of a mixture according to claims 1-2 to improve an exercise performance by a mammal, wherein a single dose of said mixture is orally administered once before the start of said exercise.
4. Use of a mixture according to claim 3, wherein said single dose of said mixture is administered 3 hours or less before the start of said exercise.
5. Use of a mixture according to claim 3, wherein said single dose of said mixture is administered 1 hour or less before the start of said exercise.
6. Use of a mixture according to claim 3, wherein said single dose of said mixture is administered about 40 minutes before the start of said exercise.
7. Use of a mixture according to any of claims 3-6, wherein said dose is more than 20mg/kg body weight of carnosine and anserine combined.
8. Use of a mixture according to any of claims 3-6, wherein said dose is at least 40mg/kg body weight of carnosine and anserine combined.
9. Use of a mixture according to any of claims 3-8 wherein said mixture is administered as a capsule.
10. Use of a mixture according to any of claims 3-9 wherein said mammal is a human.
11. Use of a mixture according to claim 10 wherein said human is an athlete and wherein said exercise is a single or repeated high-intensity exercise.

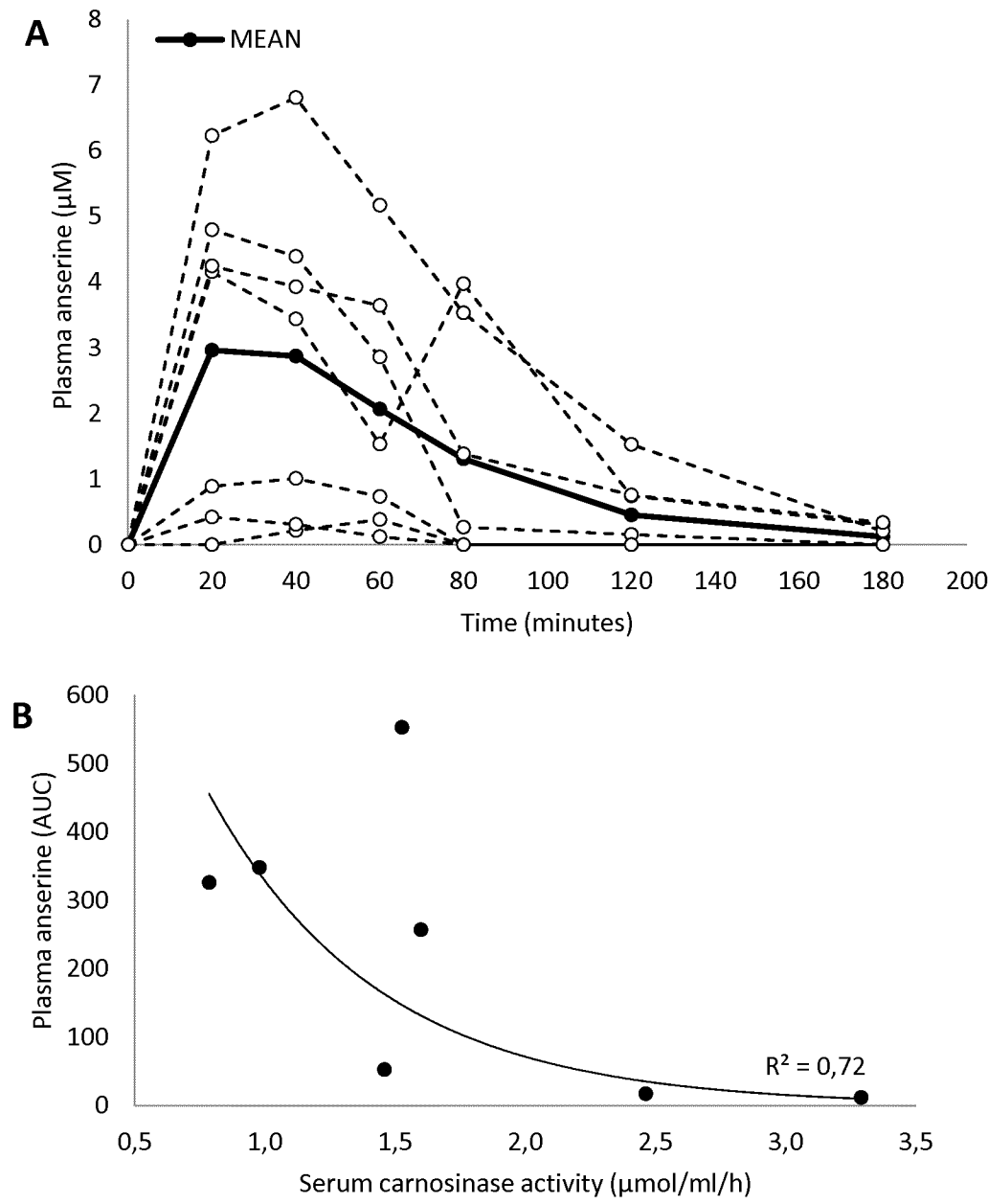


Figure 1

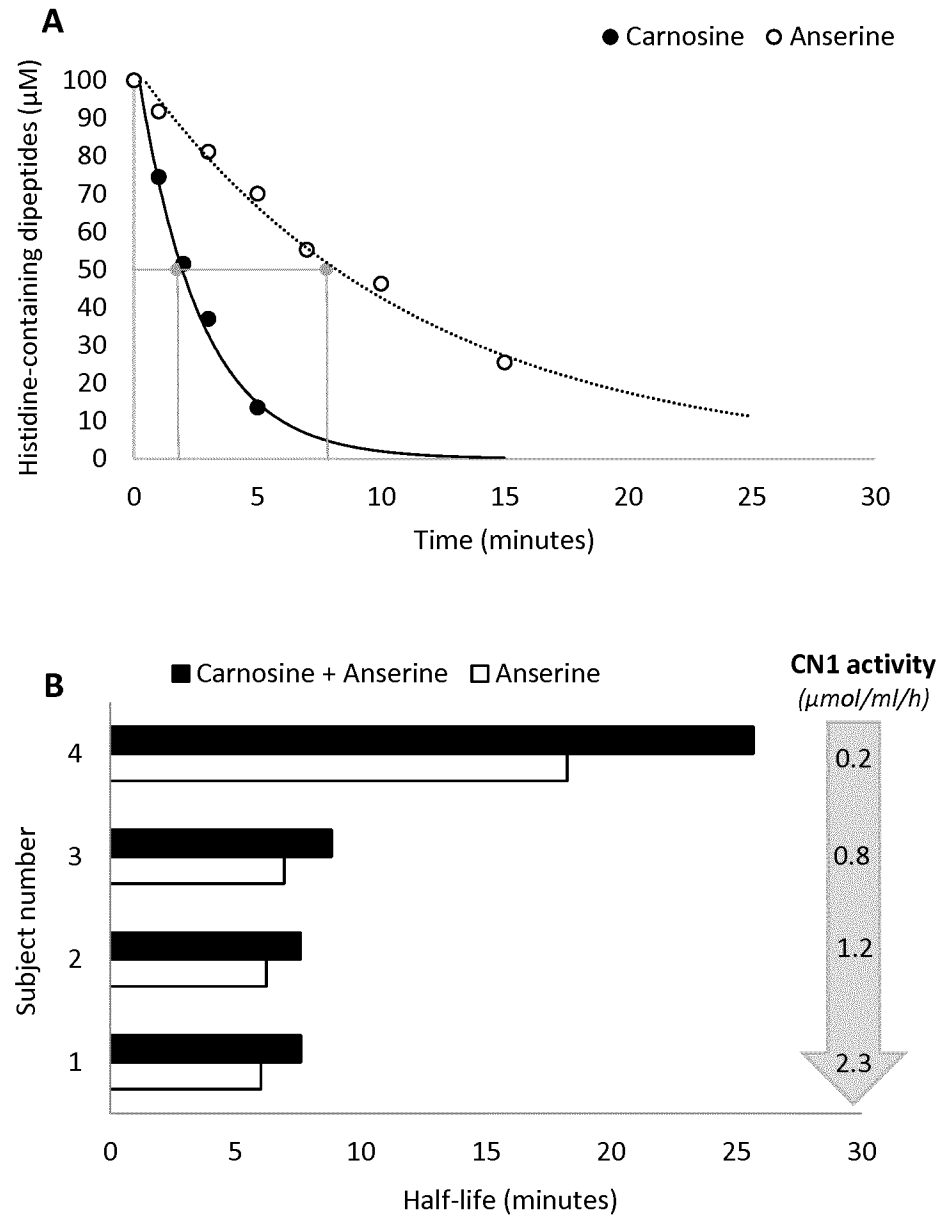


Figure 2

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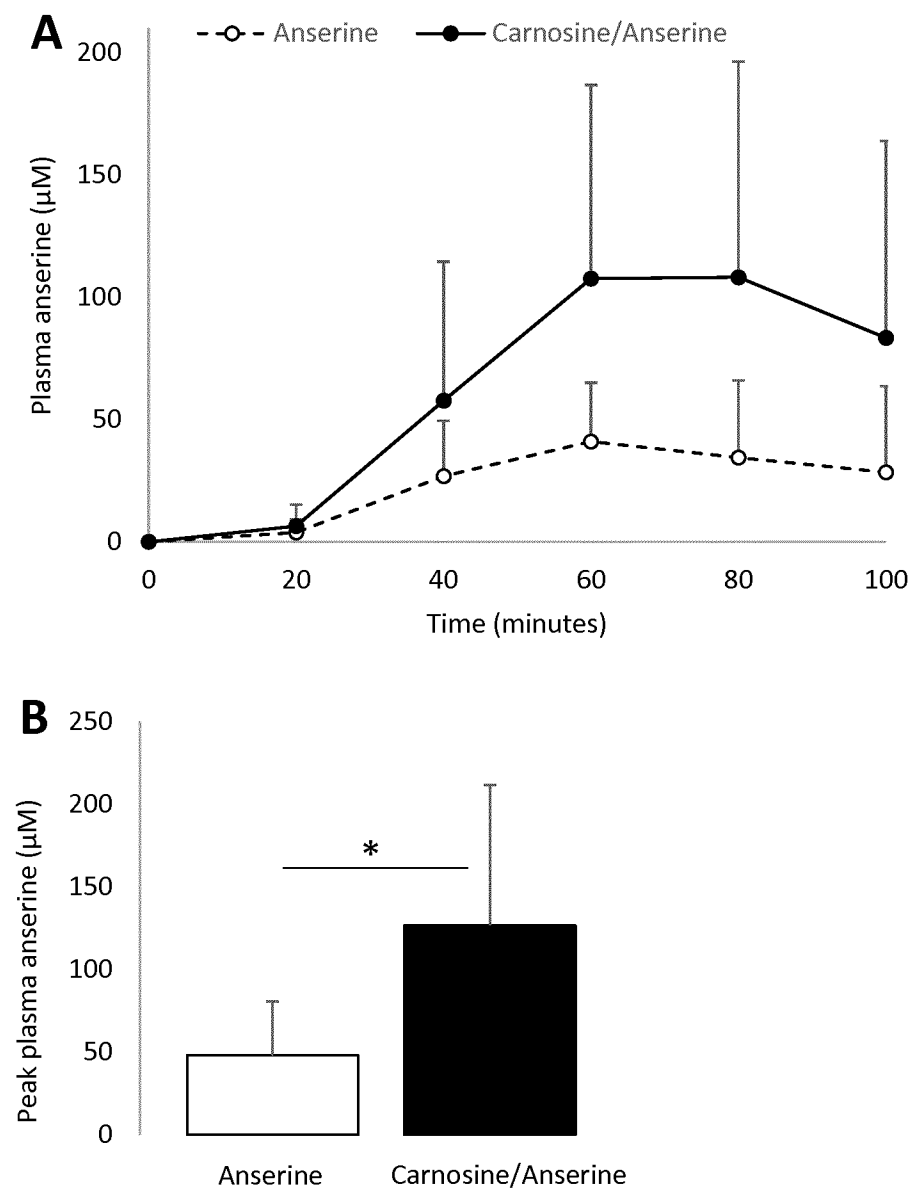


Figure 3

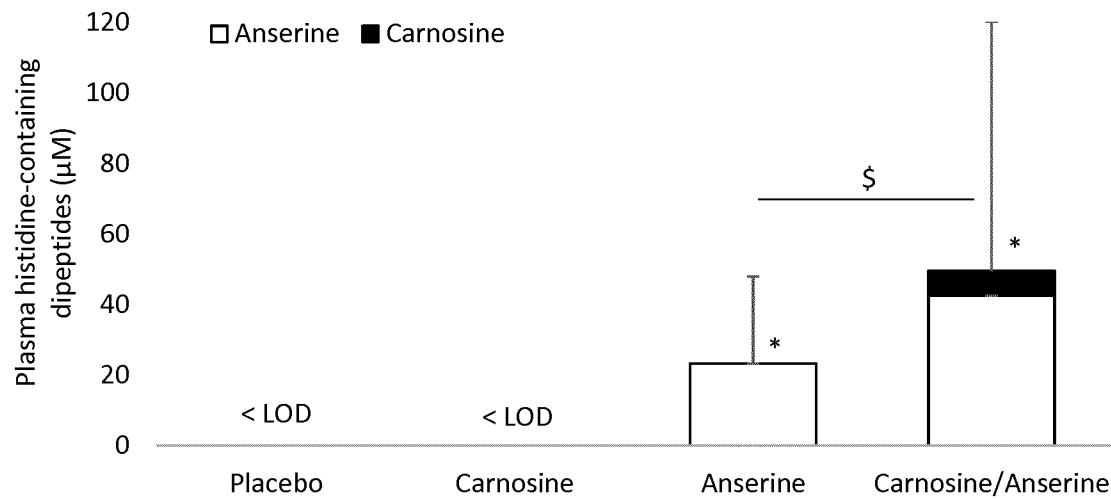


Figure 4

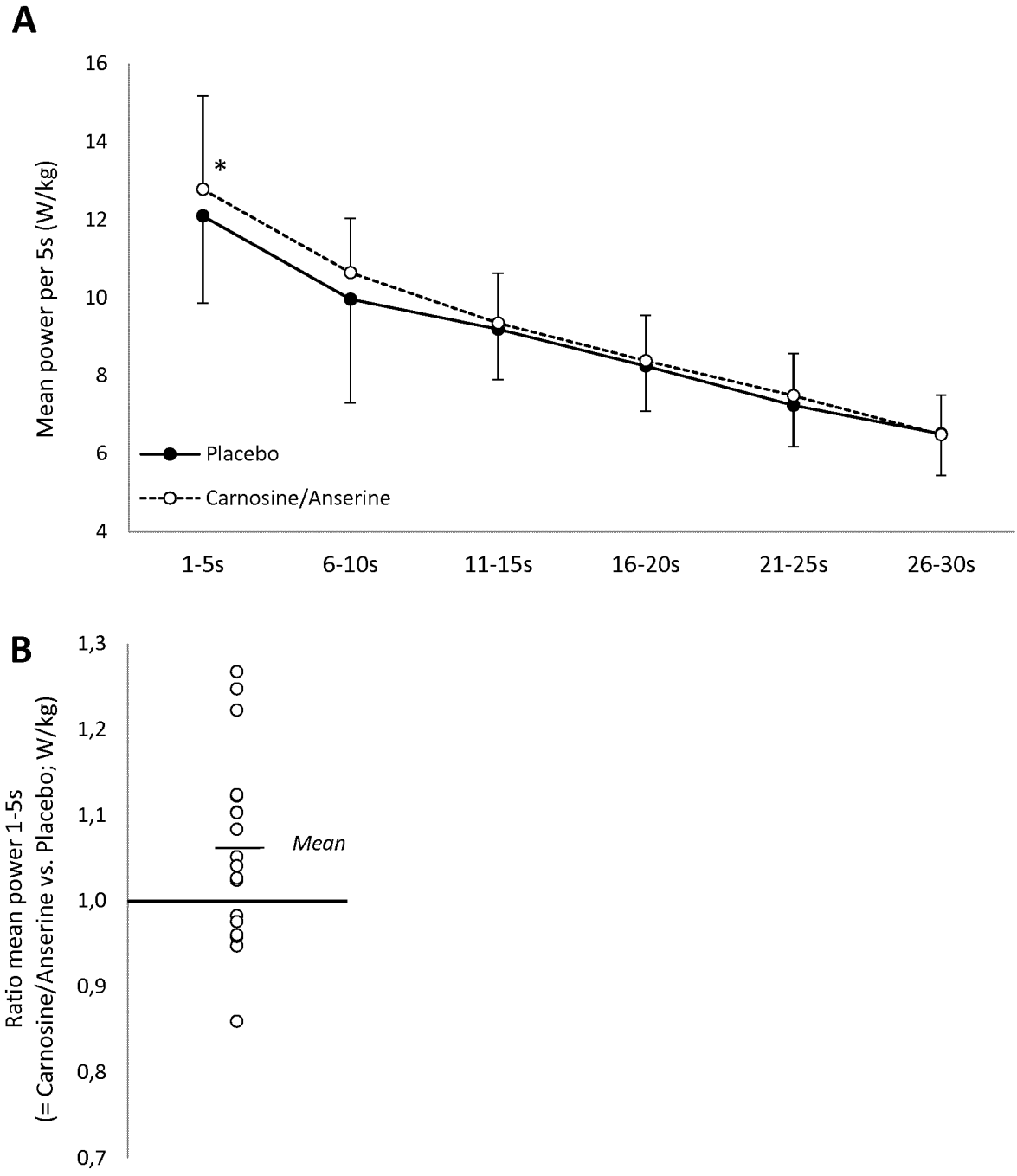


Figure 5

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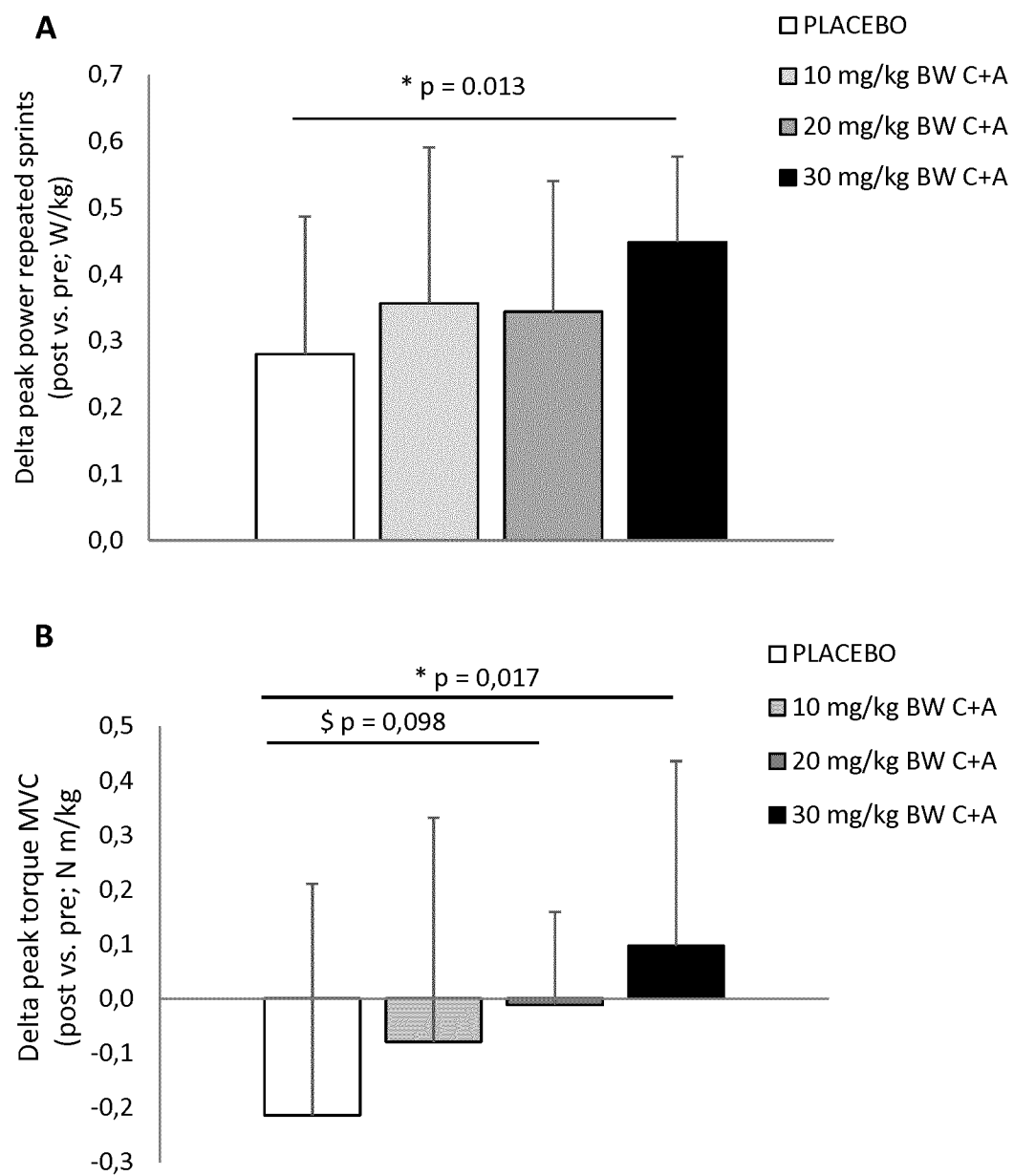


Figure 6

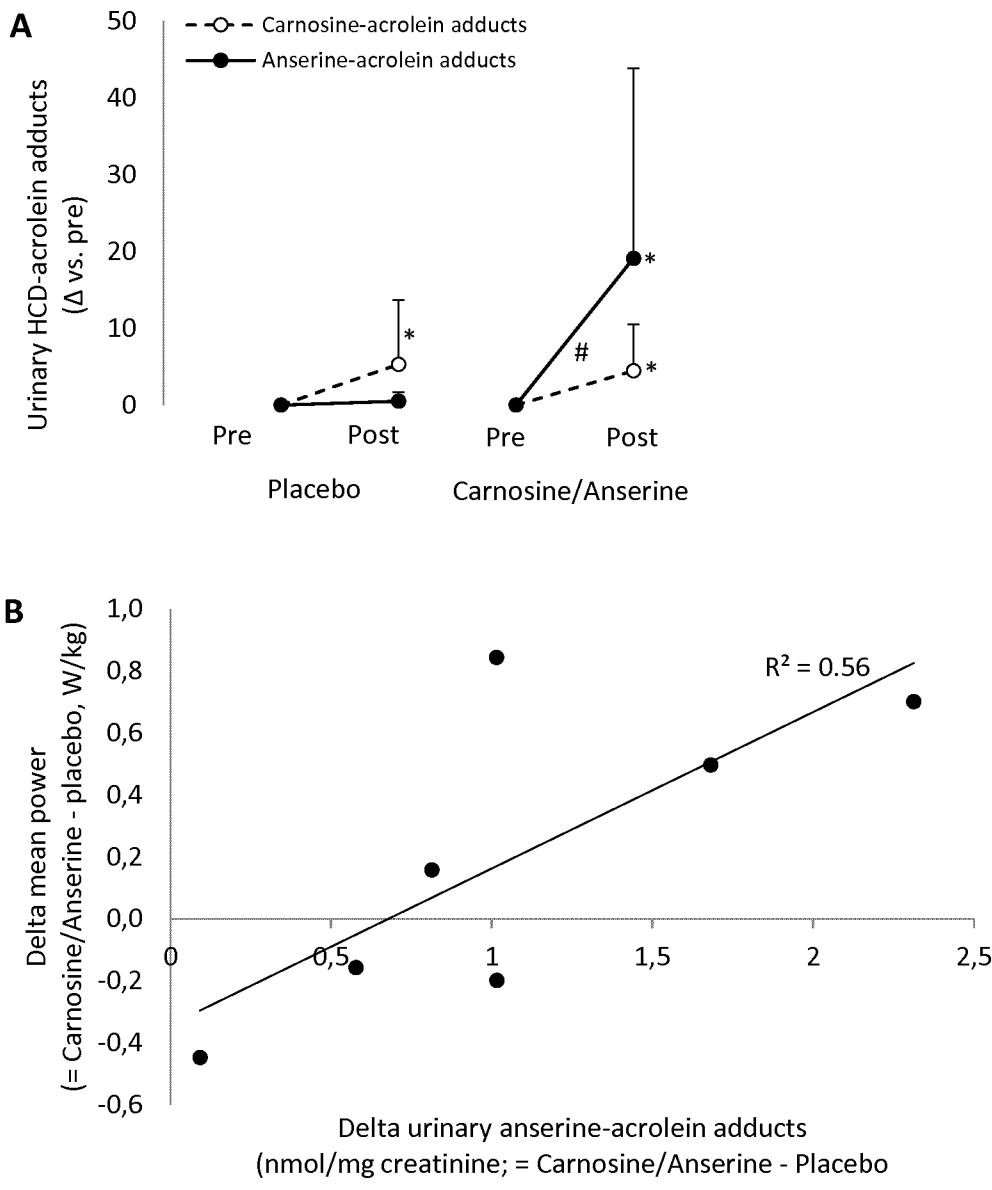


Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/084075

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K38/05 A23L33/17 A23L33/18 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K A23L		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, FSTA, BIOSIS, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 210 940 A2 (YAIZU SUISANKAGAKU IND CO LTD [JP]) 5 June 2002 (2002-06-05) cited in the application claims paragraphs [0002], [0008], [0013], [0015], [0016]	1-11
X	----- US 2009/087495 A1 (TOYOMURA KOJI [JP] ET AL) 2 April 2009 (2009-04-02) cited in the application claims paragraphs [0002] - [0040]	1-11
X	----- JP 2002 051730 A (NIPPON MEAT PACKERS) 19 February 2002 (2002-02-19) abstract ----- -/-	1-11
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 15 February 2018		Date of mailing of the international search report 26/02/2018
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Popa, Marian

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	US 2013/096193 A1 (KNELLER BRUCE W [US]) 18 April 2013 (2013-04-18) claims; examples paragraphs [0030] - [0037] -----	1-11
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