UNIVERSITEIT GENT

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

MUSCLE CARNOSINE HOMEOSTASIS: A UNIQUE SET OF REGULATORY MECHANISMS

Laura BLANCQUAERT

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Health Sciences

GHENT 2016

Supervisor:

Prof. Dr. Wim Derave (UGent)

Co-supervisor:

Dr. Inge Everaert (Ugent)

Supervisory board:

Prof. Dr. Wim Derave

Prof. Dr. Jan Bourgois

Prof. Dr. Martine Thomis

Examination board:

Prof. Dr. Stefaan De Smet (UGent)

Dr. Nathalie Michels (UGent)

Prof. Dr. Jan Boone (UGent)

Dr. Maria Veiga-da-Cunha (Université catholique de Louvain)

Prof. Dr. Giancarlo Aldini (University of Milan)

Prof. Dr. Dirk De Clercq (UGent)

Acknowledgement:

The research reported in this thesis was supported by Research Foundation Flanders (FWO) (promotor W. Derave).

Department of Movement and Sports Sciences, Watersportlaan 2, 9000 Ghent, Belgium.

Printed by University Press.

Dankwoord - Acknowledgements

Met een klein hartje, zo begon ik aan mijn eerste werkervaring in het HILO. Geen idee wat ik moest verwachten, maar vanaf dag één werd ik er ingesmeten. Een overmaat aan informatie en tal van vragen en onzekerheden kwamen de eerste maanden op me af. Vandaag, een dikke 4 jaar later, kan ik met volle overtuiging zeggen dat het een heel leerrijke en plezierige periode is geweest. Al verliep het niet altijd van een leien dakje en was het een proces van vallen en opstaan. Ik ben dan ook heel blij dat ik hier nu sta en de kans krijg om alle mensen te bedanken die me gedurende deze periode gesteund of geholpen hebben op eender welk manier.

Eerst en vooral wil ik graag de jury bedanken. Thank you for reading my thesis and providing your valuable suggestions and critical insights. Your feedback definitely helped to improve my thesis.

Wim, zonder jou had ik hier natuurlijk niet gestaan. Na mijn studies heb je me de kans gegeven om 3 maand te proeven van wat een doctoraat juist inhoudt en ik ben blij dat ik daarna definitief deel kon uitmaken van TeamDerave. Met jouw laaiend enthousiasme en onnoemlijk grote kennis slaagde je er telkens opnieuw in om me te boeien en nieuwe inzichten bij te brengen. Maar naast onze big boss ben jij ook iemand die graag eens een stapje in de wereld zet en ontspanning perfect weet te combineren met inspanning. Ik ben er rotsvast van overtuigd dat deze combinatie de drijvende kracht is achter het team. Ik heb de voorbije jaren enorm veel van jou geleerd, zowel op als naast het werk. Ik ben blij dat ik je op z'n minst iets kon bijbrengen over het gebruik van whatsapp en snapchat ☺ Dank je wel voor je begeleiding, je schouderklopjes als het eens nodig was, en je geloof in mijn kunnen.

De bureau zou natuurlijk de bureau niet zijn zonder mijn lieftallige collega's. Toen ik startte, was de eerste generatie van TeamDerave nog aanwezig in het HILO. Sanne, Audrey en Inge, jullie hebben er voor gezorgd dat ik me direct thuis voelde in het team. Sanne, jouw enthousiasme werkte zo aanstekelijk en je gedrevenheid is enorm. Audrey, je nauwkeurigheid en efficiëntie zijn onnavolgbaar. Ik heb veel van jou geleerd en opgestoken. Bedankt voor je eindeloze hulp en steun. Sanne en Audrey, jullie worden gemist op de bureau! Inge, miniboss, als er iemand is die me alle kneepjes van het vak geleerd heeft, ben jij het wel. Van HPLC tot PCR en het vastnemen van mijn eerste muis, jij was steeds mijn coach van het eerste uur. Ik heb je geduld meer dan eens op de proef gesteld met mijn soms ietwat tragere handelingen of getreuzel, maar je hebt steeds met een glimlach al mijn vragen beantwoord. Je nuchtere kijk op dingen in combinatie met je bereidwilligheid om overal te helpen maken van jou de ideale coach. Bedankt voor al je hulp, of je het nu leuk vindt of niet, voor mij ben en blijf je de enige echte miniboss. Helene, ook jij was er bij vanaf het eerste uur. Een bezoekje aan het UZ was altijd aangenamer met een gezellige babbel bij jou. Ik heb enorm veel respect voor de manier

waarop jij je doctoraat hebt afgewerkt. Bedankt voor je vele labotips en leuke HILO bezoekjes.

Toen ik aan het HILO begon, was mijn thesispartner al een jaar deel van TeamDerave. Tine, over onze vriendschap kan ik een doctoraat op zich schrijven. Het is dus onmogelijk om in enkele zinnetjes te verwoorden wat jouw steun voor mij betekent. Je was altijd bereid om me raad te geven en je tips & tricks kwamen altijd van pas. Maar ook naast het werk kan ik met alles bij jou terecht. Ik zal je echt missen in de komende maanden op het HILO, maar ik weet nu al met 100% zekerheid dat je het er uitstekend zal vanaf brengen in je nieuwe uitdagingen. Jan, onze eerste man in TeamDerave, onze vreemde eend in de bijt ☺ Je enthousiasme en passie voor onderzoek sieren je en ik heb veel bewondering voor wie je bent. Onze dagelijkse tête-à-têtes en gezellige babbels op de bureau zijn altijd heel ontspannend. Ik ben er zeker van dat je een mooi doctoraat zal afleggen en belangrijker nog, een fantastische papa zal zijn voor je zoon. Eline en Maxime, jullie zijn de laatste aanwinsten in ons team. Eline, je gedrevenheid om alles goed te doen en je vastberadenheid om je doelen te bereiken, getuige daarvan de marathon die je met glans hebt uitgelopen, kenmerken je. Maar daarnaast ben jij ook een heel joviaal en sociaal iemand met zin voor avontuur. Ik kan alvast niet wachten om samen te vertrekken naar Nieuw-Zeeland! Maxime, ook jou wil ik graag bedanken voor de toffe bureaumomenten en je opgewektheid. Je leergierigheid en analytische kennis zullen je zeker en vast ver brengen. Anneke, ook jou wil ik bedanken voor de vele hulp bij analyses en labotesten.

Ik wil ook heel wat andere collega's bedanken die mijn tijd aan het HILO verrijkt hebben. Bert, Margot, Sofie en Sara, bedankt voor de leuke bureaumomenten tijdens de beginperiode van mijn doctoraat. Bert, jou wil ik ernaast ook bedanken voor je begeleiding tijdens onze thesis. Je enthousiasme voor het onderzoek heeft deels bijgedragen aan mijn keuze om te starten met een doctoraat. Lieze en Jasmien, het is leuk dat jullie als ex-studiegenoten ook het HILO kwamen vervoegen en we zo collega-vrienden werden. Bedankt voor alle leuke loopjes en babbels. Ook de jonge HILO garde wil ik graag bedanken voor hun frisse (en soms iets minder frisse) wind op onze gang. Kevin, Kobe, Janne, Senne, Maarten en Rud, bedankt voor jullie jeugdig enthousiasme en de ontspannende uitjes in de laatste en soms wat stresserende periode van mijn doctoraat. Ten slotte wil ik ook graag alle andere collega's bedanken voor de spetterende HILOweekends, zalige middagloopjes, lekkere traktaties en topafterworks. Alle collega's dragen bij aan de fantastische werkplek die het HILO is, bedankt daarvoor.

Daarnaast ook een grote dankjewel aan alle vrienden en vriendinnen die me steunen en de ontspannende momenten zo leuk maken. Stien, Jolien, Geraldine en Berdien, na meer dan 20 jaar vriendschap kunnen we zeggen dat we elkaar door en door kennen. Ook al zijn we nu elk onze eigen weg ingeslaan, onze uitjes blijven altijd op en top leuk en gezellig. Jullie liggen me heel nauw aan het hart en ik hoop dat onze levenslange vriendschap ons nog mooie momenten mag bezorgen. Ook de LO ladies en LO boys wil ik

graag bedanken voor de onvergetelijke studententijd die we samen hebben beleefd. Onze jaarlijkse reünie op de proclamatie is al 5 jaar een traditie en ik hoop dat er nog vele jaren volgen. Mijn favoriete voetbalvrouwen, Tiffany en Liesbeth, ook jullie bedankt voor de gezellige etentjes en knusse uitjes. Verder wil ik nog de girls-'squad' bedanken voor de vele avondjes uit, de gezellige verjaardagsdrinks die steevast eindigen in een feestje, de hilarische Ardennenweekendjes, en ja, zelfs de chaotische verkleed en themafeestjes. Al deze momenten vormden de ideale ontspanning en uitlaatklep tijdens mijn doctoraatsperiode. Julie, ik ben blij dat je naast een vriendin, nu ook een collega bent geworden. Een speciaal woordje van dank voor Eline en Eveline, my happy two friends. Bedankt voor jullie vriendschap en de keer op keer leuke uitjes. Eveline, je creativiteit is bewonderenswaardig, getuige daarvan de mooie tekening op de cover van mijn boekje. Ik wil je dan ook enorm bedanken om dit te maken. Eline, ik ben blij dat we na de LO jaren dichter naar elkaar zijn toegegroeid. Een leuke shopnamiddag, een gezellig loopje, een topfeestje, we kunnen alles delen en ik hoop dat we dat nog lang kunnen doen.

Naast al deze vrienden wil ik ook heel graag mijn familie bedanken. In de eerste plaats mijn schoonfamilie, Paul, Els, Laurens en Jeannine. Bedankt voor jullie steun en interesse in alles wat ik doe. Jullie warme karakters en eindeloze steun zorgen ervoor dat ik me helemaal thuis voel bij jullie. Ik wil jullie dan ook enorm bedanken voor alles wat jullie al voor ons gedaan hebben.

Kenneth, Elien en kleine Remi, ook jullie bedankt voor jullie steun. Kenneth, we kunnen zeggen dat we twee uiteenlopende karakters zijn. In iedere familie is er ten slotte wel een brave en iets minder brave zoon of dochter ☺ Maar ondanks onze verschillende levens zal ja altijd mijn grote broer zijn en ik ben heel fier op wat je doet. Samen met Elien en Remi vormen jullie een heel mooi gezinnetje.

Mama en papa, bedankt voor jullie onvoorwaardelijke steun. Als kind hebben jullie me steeds alle kansen gegeven, inclusief alle sporten die ik wou uitproberen en de vele ritjes die daarmee gepaard gingen. En dat heeft er mede voor gezorgd dat ik hier nu sta. Bedankt om telkens klaar te staan waar nodig en me de volle 100% te steunen in al mijn keuzes.

Dan rest er me nog één speciaal iemand. Lieverd, waar zal ik beginnen? Jij staat altijd voor me klaar en steunt me door dik en dun. Nog even wat stalen oppikken in het labo, snel eens passeren in het UZ of me in het weekend vergezellen naar Merelbeke, niets was jou te veel gevraagd, ook al kwamen we daardoor vaak al eens ergens te laat ☺ Ik zou niet weten waar te beginnen zonder jou en kan niet wachten om samen onze toekomst verder uit te bouwen.

Table of contents

List of abbreviations

AGXT2	alanine-glyoxylate transaminase
AOA	aminooxyacetate
ANS	anserine
BA	beta-alanine
BAIBA	beta-aminoisobutyric acid
BW	body weight
Ca^{2+}	calcium
CARN	carnosine
CARNS	carnosine synthase enzyme
CNDP1	gene encoding human serum carnosinase enzyme
CNDP2	gene encoding cytosolic non-specific dipeptidase
CN1	human serum carnosinase or carnosine dipeptidase 1
CN2	cytosolic non-specific dipeptidase or carnosine dipeptidase 2
DW	drinking water
GABA	gamma-aminobutyric acid
GABA-T	4-aminobutyrate-2-oxoglutarate transaminase
GADL1	glutamate decarboxylase like protein 1
GASTR	gastrocnemius
H^+	proton
HCD	histidine-containing dipeptide
HDC	Histidine decarboxylase enzyme
HIS	histidine
^{1}H-MRS	proton magnetic resonance spectroscopy
HPLC	high-performance liquid chromatography
mRNA	messenger RNA
MSA	malonate semi-aldehyde
PAT1	proton-coupled amino acid transporter 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEPT1	peptide transporter 1
PEPT2	peptide transporter 2
PHT1	peptide/histidine transporter 1
PHT2	peptide/histidine transporter 2
POT	proton coupled oligopeptide transporters
SAL	saline
SSA	succinate semi-aldehyde
TA	tibialis anterior
TauT	taurine transporter
TTE	time to exhaustion
VIG	vigabatrin

English summary

In the past decades, dietary supplements have gained in popularity worldwide. Athletes are a specific population that constantly search for strategies to improve performance. In this population, supplements can support the body during and after the hard periods of training and thereby increase exercise performance. However, the efficacy of only a few supplements is supported by well substantiated evidence, and one such supplement is beta-alanine. Beta-alanine is able to increase intramuscular carnosine concentrations. This carnosine loading may thereby augment fatigue threshold and improve high-intensity exercise performance, as several physiological roles are ascribed to the dipeptide (pH-buffering, calcium regulation, antioxidant capacities). Because of the range of functions carnosine exerts, it is also a promising dipeptide for some health-related issues.

Carnosine is a naturally occurring dipeptide with a high concentration in mammalian skeletal muscle. It is synthesized by carnosine synthase from the amino acids L-histidine and beta-alanine. Muscle carnosine concentrations are shown to be highly stable over time, suggesting that carnosine is subject to a strong homeostatic regulation keeping carnosine levels within a certain normal range. However, as mentioned, one condition in which muscle carnosine homeostasis is greatly disrupted is beta-alanine supplementation. Several studies demonstrated that chronic oral ingestion of beta-alanine can substantially elevate the carnosine content by 40-80%, which subsequently leads to improved performance in high intensity exercise in both trained and untrained individuals. Because of the popularity of beta-alanine as a supplement and the beneficial effects of high muscle carnosine levels, it is important to have a full understanding of the carnosine metabolism and the regulation of muscle carnosine homeostasis.

Although beta-alanine is a frequently used dietary supplement, it was recently demonstrated that only 2-3% of the total ingested amount of beta-alanine is actually incorporated into muscle carnosine. This indicates that the major part of ingested beta-alanine has an unknown metabolic fate, signifying that the beta-alanine and carnosine

metabolism are not yet fully unraveled and may include a complex regulation of a set of enzymes and transporters.

Study 1 of this thesis mainly focused on the role of beta-alanine transaminases in the regulation of muscle carnosine levels upon beta-alanine supplementation. Because most chronically ingested beta-alanine has an unknown metabolic fate, a possible pathway is transamination by GABA-T and AGXT2 in either liver and/or kidney or inside myocytes. GABA-T and AGXT2 were shown to be mainly expressed in kidney and liver and to a much smaller extent in myocytes, suggesting that beta-alanine transamination mainly takes place in these organs. By inactivating the beta-alanine transaminase pathways, both higher circulating beta-alanine levels and higher muscle carnosine loading could be evoked. Thus, muscle carnosine homeostasis is shown to be dependent on the circulating availability of beta-alanine, which is in turn dependent on the degradation of beta-alanine in liver and kidney. These findings partly explain the low efficiency of chronically ingested beta-alanine because beta-alanine is primarily routed toward oxidation. Only upon saturation of this pathway, beta-alanine is incorporated in muscle carnosine.

In **study 2**, focus was shifted to the other amino acid involved in carnosine synthesis. As the efficiency of beta-alanine supplementation is low, it can be questioned whether beta-alanine is indeed the one and only rate-limiting factor for carnosine synthesis and whether carnosine loading efficiency can be enhanced by L-histidine supplementation (alone or combined with beta-alanine). The results indicated that muscle carnosine is not enhanced by L-histidine supplementation, confirming the rate-limiting role of beta-alanine in the carnosine synthesis process. However, chronic beta-alanine supplementation was shown to reduce plasma and muscle histidine levels, demonstrating that, although not rate-limiting, L-histidine availability is not unlimited either. The decline is body histidine levels could be prevented by co-supplementing L-histidine alongside beta-alanine. Further research on the effect of the depletion of histidine levels by beta-alanine supplementation on physiological processes such as carnosine loading of longer duration or protein synthesis in an anabolic state is necessary.

To investigate whether muscle carnosine homeostasis is equally disrupted by the absence of any dietary beta-alanine, **study 3** was performed. Because meat and fish are the main

exogenous source of carnosine, the effect of a 6-month vegetarian diet in previous omnivorous subjects on the carnosine homeostasis was examined. Next to carnosine, creatine and carnitine were also monitored in this study. It was demonstrated that body creatine, but not carnosine and carnitine homeostasis was affected by the 6-month vegetarian diet. These findings suggest that carnosine and carnitine homeostasis can be effectively maintained by endogenous synthesis of these compounds or their precursors.

Lastly, **study 4** explored the transcriptional events of carnosine-related enzymes and transporters in human skeletal muscles in response to beta-alanine supplementation in order to further elucidate how muscle carnosine homeostasis is disturbed. We found that both beta-alanine transporters and carnosine synthase were greatly upregulated, indicating that the mRNA expression of these effectors is enhanced by increased circulating beta-alanine levels. Thus, increased transsarcolemmal beta-alanine uptake and muscle carnosine synthesis can be seen as a way to maintain plasma beta-alanine homeostasis, thereby disturbing muscle carnosine homeostasis.

Altogether, this thesis provided more insights in the regulation of plasma beta-alanine and muscle carnosine homeostasis. As beta-alanine is a popular dietary supplement, a better understanding of its metabolism can lead to clearer guidelines for supplementation.

Nederlandse samenvatting

Voedingssupplementen hebben de laatste jaren enorm aan populariteit gewonnen. Een specifieke populatie die constant op zoek is naar strategieën om hun prestatie te verbeteren zijn atleten. Voor hen kunnen supplementen belangrijk zijn om het lichaam te ondersteunen tijdens en na de zware trainingsperiodes, en op die manier prestaties tot een hoger niveau te tillen. Slechts weinig supplementen hebben een grondig wetenschappelijk onderbouwde werking. Een supplement dat wel wetenschappelijk onderbouwd is, is beta-alanine. Beta-alanine is in staat om de intramusculaire concentratie van het metaboliet carnosine te verhogen. Deze carnosine oplading kan op zijn beurt prestatiebevorderende effecten uitoefenen, wat kan worden toegeschreven aan de verschillende functies die carnosine bezit (pH-buffering, calcium regulatie, antioxidante capaciteiten). Door deze veelheid aan functies heeft carnosine ook veelbelovende klinische toepassingen.

Carnosine is een natuurlijk voorkomend dipeptide waarvan hoge concentraties worden gevonden in de skeletspieren van zoogdieren. Carnosine wordt gesynthetiseerd door het enzyme carnosine synthase uit de aminozuren L-histidine en beta-alanine. Het is reeds aangetoond dat spiercarnosine concentraties stabiel zijn overheen de tijd, wat doet vermoeden dat carnosine onderhevig is aan een sterk homeostatisch systeem, dat de carnosine concentraties binnen een bepaalde range houdt. Een situatie waarin spier carnosine homeostase danig verstoord is, is chronische beta-alanine supplementatie. Verschillende studies toonden reeds aan dat de chronische inname van beta-alanine de hoeveelheid spiercarnosine kan verhogen met 40-80%, wat op zijn beurt leidt tot een verhoogd prestatievermogen bij inspanningen aan hoge intensiteit in zowel getrainde als ongetrainde individuen. Omwille van de populariteit van beta-alanine en de gunstige effecten van verhoogde spiercarnosine concentraties is het belangrijk om een goed beeld te hebben van het carnosine metabolisme en de regulatie van de spiercarnosine homeostase.

Alhoewel beta-alanine op de dag van vandaag een veel gebruikt voedingssupplement is, werd recent pas aangetoond dat slechts 2-3% van de totale ingenomen beta-alanine

werkelijk wordt omgezet in spiercarnosine. Dit toont aan dat het grootste deel een tot nu toe ongekend metabolisch lot heeft in het menselijk lichaam, wat doet vermoeden dat het beta-alanine en carnosine metabolisme nog niet volledig gekend is en het een complexe regulatie omvat waarin verschillende enzymes and transporters betrokken zijn.

Studie 1 van deze thesis focuste op de rol van beta-alanine transaminases in de regulatie van spiercarnosine concentraties na beta-alanine supplementatie. Aangezien het grootste deel van de chronisch gesupplementeerde beta-alanine een nog ongekend metabolisch lot heeft, is transaminatie door de enzymes GABA-T en AGXT2 in lever, nier of spiercellen een mogelijke alternatieve reactieweg. GABA-T en AGXT2 expressie werd voornamelijk gevonden in de nier en lever en in kleinere mate in de spiercellen, wat doet vermoeden dat beta-alanine transaminatie voornamelijk in deze organen plaatsvindt. Door het inhiberen van de beta-alanine transaminase reactieweg werden verhoogde circulerende beta-alanine concentraties en een verhoogde mate van spiercarnosine oplading gevonden. Het is dus aangetoond dat homeostase van carnosine afhankelijk is van de beschikbaarheid van beta-alanine in de circulatie, wat op zijn beurt wordt bepaald door de afbraak van beta-alanine in de lever en nieren. Deze bevindingen bieden deels een verklaring voor de lage efficiëntie van chronisch ingenomen beta-alanine, aangezien beta-alanine eerst door de transaminases wordt gemetaboliseerd. Enkel wanneer deze reactieweg gesatureerd is, wordt beta-alanine gebruikt voor carnosine synthese.

In **studie 2** werd de focus verlegd naar het andere aminozuur dat betrokken is bij de synthesis van carnosine, zijnde histidine. Aangezien de efficiëntie van beta-alanine supplementatie laag is, kan het immers in vraag worden gesteld of beta-alanine wel degelijk de enige snelheidsbepalende factor is van het carnosine synthese proces en of de efficiëntie van carnosine oplading verhoogd kan worden door L-histidine supplementatie (alleen of gecombineerd met beta-alanine). L-histidine supplementatie leidde echter niet tot verhoogde spiercarnosine, wat de snelheidslimiterende rol van beta-alanine bevestigt. Chronische beta-alanine supplementatie leidde echter wel tot een daling van de plasma en spier histidine concentraties, wat aantoont dat de L-histidine beschikbaarheid niet ongelimiteerd is. Deze daling in histidine concentraties werd tegengegaan door de co-supplementatie van beta-alanine met L-histidine, maar verder onderzoek is nodig om het effect van gedepleteerde histidine reservoirs door beta-alanine supplementatie op

fysiologische processen zoals spiercarnosine oplading van langere duur en eiwitsynthese uit te spitten.

Om na te gaan of spiercarnosine homeostase in eenzelfde mate verstoord kan worden door de afwezigheid van exogene beta-alanine, werd **studie 3** uitgevoerd. Aangezien vlees en vis de belangrijkste exogene bron zijn van carnosine, werd het effect van een 6 maand vegetarisch dieet in omnivore personen onderzocht op de homeostase van 3 zogenaamde carninutriënten, zijnde carnosine, carnitine en creatine. Deze studie toonde aan dat creatine homeostase, maar niet carnosine en carnitine homeostase, verstoord wordt door een 6 maand vegetarisch dieet. Deze bevindingen geven aan dat carnosine en carnitine homeostase vermoedelijk behouden blijven door voldoende endogene synthese van deze metabolieten of hun precursoren.

Tenslotte onderzocht **studie 4** de transcriptionele gebeurtenissen van carnosine gerelateerde enzymes en transporters in de humane skeletspier na beta-alanine supplementatie om zo te achterhalen hoe de spiercarnosine homeostase verstoord wordt. Zowel beta-alanine transporters als carnosine synthese is in grote mate opgereguleerd wat aantoont dat de mRNA expressie van deze effectoren verhoogd is door verhoogde circulerende beta-alanine concentraties. Verhoogde beta-alanine opname in de spier en carnosine synthese kan dus gezien worden als een manier om circulerende beta-alanine homeostase te behouden, wat op zijn beurt leidt tot verstoorde spiercarnosine homeostase.

Samenvattend kunnen we stellen dat dit doctoraat bijgedragen heeft aan een verhoogde kennis van de regulatie van circulerend beta-alanine en spier carnosine. Door de populariteit van beta-alanine als een voedingssupplement kan deze verhoogde kennis bijdragen aan betere richtlijnen voor supplementatie.

I

General introduction

Parts of the introduction are based on the review of Blancquaert et al. (2015)

1. Introduction

The human body is a complex system with many interacting parts controlled by a set of regulatory mechanisms. In a constantly changing external environment, regulatory mechanisms support the body to adapt to these changes and thereby maintain constancy of the internal environment (*milieu intérieur*). The originator of this concept is the French physiologist Claude Bernard. Shortly after, the American physiologist Walter Cannon coined the term homeostasis (homeo: unchanging + stasis: standing) to describe this process of internal constancy (Cannon, 1929).

Countless processes in the human body are subject to homeostatic regulation, such as the core body temperature, blood pressure, blood glucose concentrations, blood pH, the volume of body water,… and any homeostatic imbalance can result in disease. The internal environment is never absolutely constant, making homeostasis a **dynamic equilibrium** in which conditions are stabilized within a normal range above and below a certain **set point**. Sensors are able to detect deviations from this set point, which is then relayed to an integrating center and ultimately activate effectors that *reverse* the detected deviation from the set point. This process is also defined as **negative feedback loops** (Fig 1).

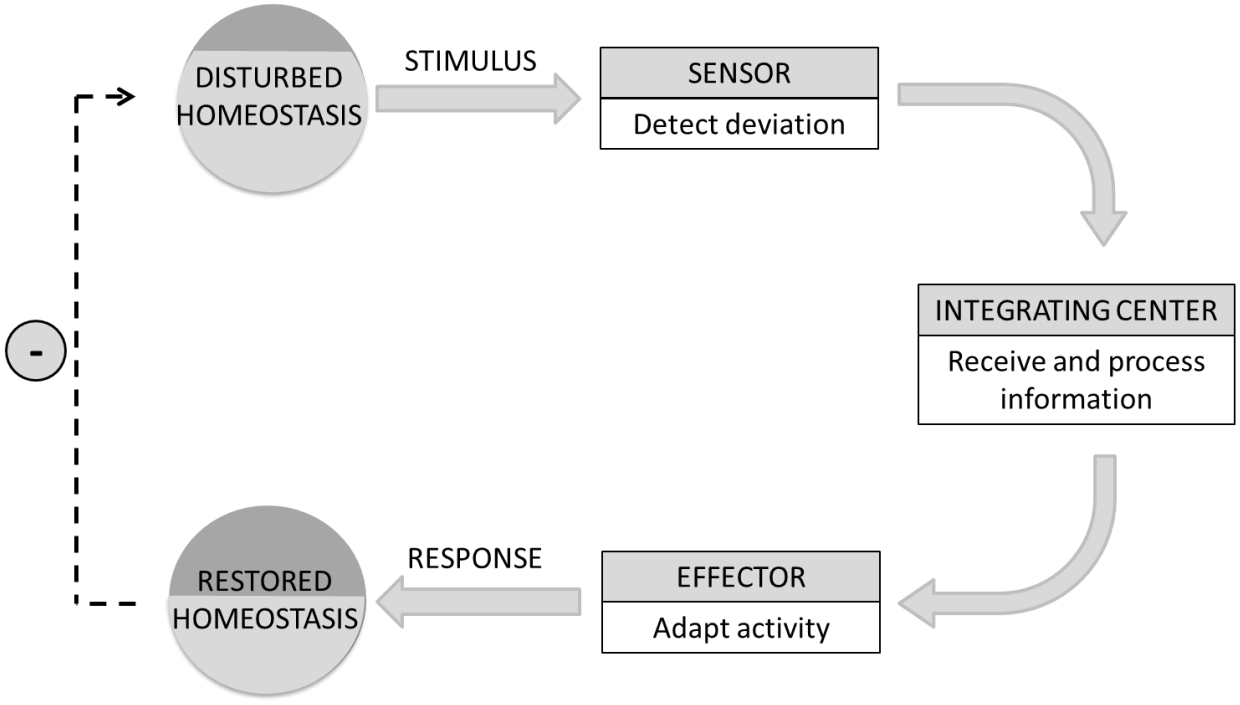

Figure 1: Graphical overview of the concept of homeostasis. Negative feedback loops (indicated by the dashed arrow) maintain a state of dynamic constancy of the internal environment

One system that was developed in the course of evolution and is under homeostatic control is the **carnosine system**, a complex and energy-consuming molecular system. Carnosine is a pleiotropic molecule and one of the most abundant metabolites in muscle cells. Different biochemical, physiological and therapeutic properties are ascribed to carnosine, making it an intriguing metabolite to investigate. This thesis will attempt to unravel body and more specifically muscle carnosine metabolism thereby helping to understand carnosine's complex homeostatic regulation.

2. Basic information on carnosine

Carnosine is the main molecule investigated in the papers of this thesis. Although carnosine has been discovered more than a century ago, this molecule gained popularity in scientific research during the past decades. In this first section, carnosine and related compounds will be described and their main physiological pathways and biochemical properties will be clarified.

2.1. Carnosine and related compounds

Carnosine was discovered in the early 1900s by the quest of the Russian chemist Vladimir Gulewitch for nitrogen-containing non-protein compounds in meat extract. Gulewitch identified two of these unknown substances that were present in rather high amounts as carnosine and carnitine (from the latin *carnis* – meat) and further focused his research on carnosine as it is one of the most abundant intramuscular molecular compounds (Boldyrev, 2012).

Carnosine is a cytoplasmic dipeptide combining the proteinogenic amino acid L-histidine with the non-proteinogenic beta-amino acid beta-alanine. Different carnosine derivatives are known of which the methylated analogs anserine and ophidine (balenine) are the most common ones. Methylation on either the pi or tau nitrogen of the imidazole ring of L-histidine forms **anserine** (beta-alanyl-Nπ-methylhistidine) or **ophidine** (beta-alanyl-Nτ-methylhistidine), respectively. Carnosine, anserine and ophidine are collectively called **histidine-containing dipeptides** (HCDs). HCDs are mainly present in mammalian skeletal muscle and neuronal tissue and to a smaller extent in the heart, liver and kidney (Boldyrev *et al.*, 2013). Carnosine is the only HCD present in human skeletal muscle (5-8mmol/l wet weight), whereas the muscles of almost all other mammals contain both carnosine along with one of the methylated analogs (anserine or ophidine). Next to the methylated analogs, **homocarnosine** is another HCD which is predominantly present in brain structures and originates from the substitution of beta-alanine by ɣ-aminobutyric acid (GABA). GABA is a non-proteinogenic amino acid and an inhibitory neurotransmitter in the brain, of wich the metabolism closely resembles beta-alanine (Boldyrev & Severin, 1990) (Fig 2).

Another non-proteinogenic compound that is somewhat related to carnosine metabolism is **taurine**. Taurine is often wrongly labeled as an amino acid but since it contains a sulphonate group instead of a carboxylgroup, it is strictly taken an amino sulfon acid. Taurine and beta-alanine have very similar molecular structures and their metabolism is intimately linked, as they share the same transporter (taurine transporter, TauT) for transsarcolemmal uptake (Harris *et al.*, 2006). Taurine is abundant in the cells of many tissues, including heart and skeletal muscles and taurine is believed to be involved in many cellular processes but in skeletal muscle its main roles are to facilitate Ca^{2+} dependent excitation-contraction processes and aid in antioxidant defense from stress responses (Spriet & Whitfield, 2015). Although it is the most abundant free amino compound in skeletal muscle, taurine is, alike beta-alanine, not incorporated into protein.

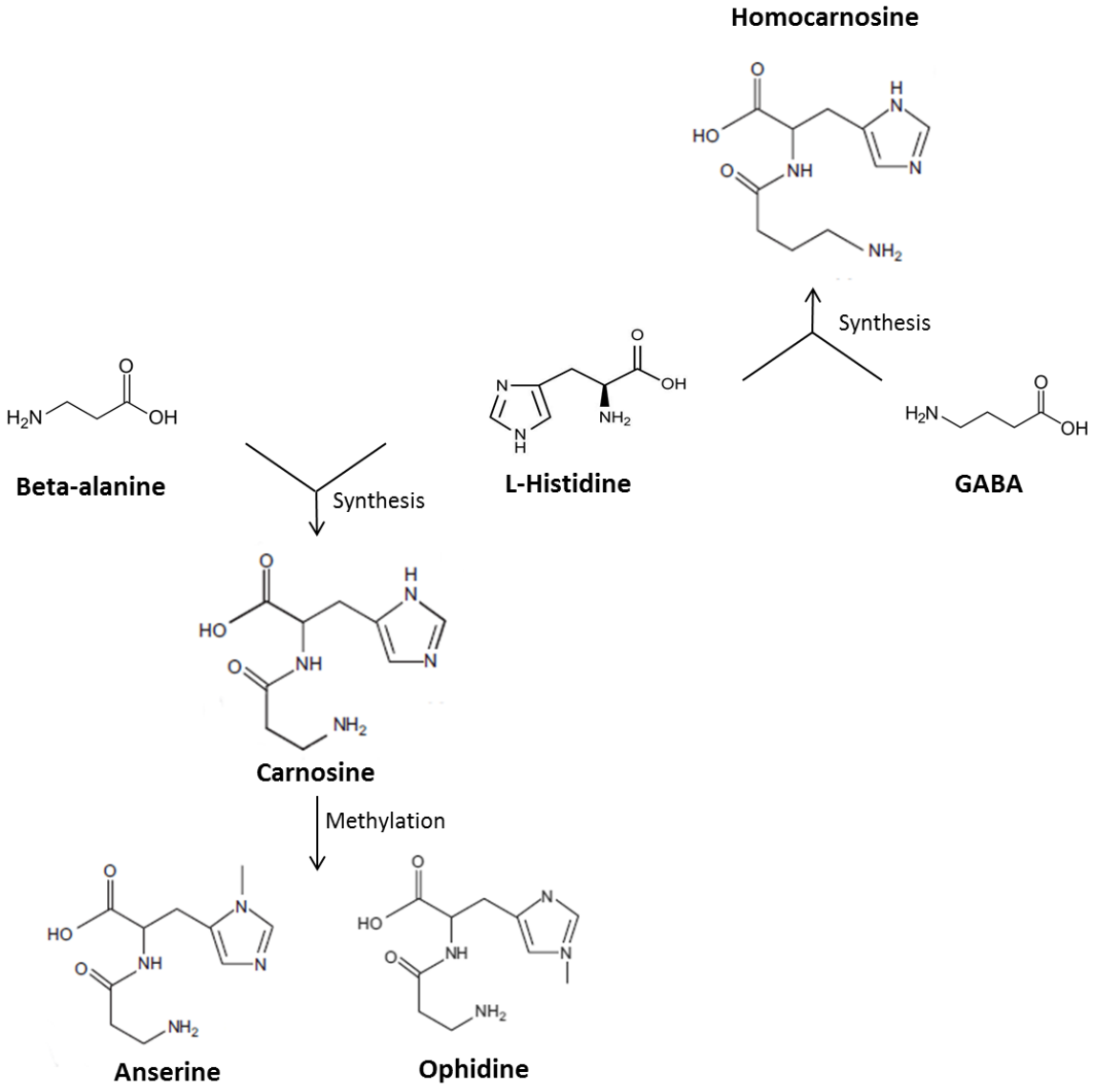

Figure 2: Carnosine and related compounds

2.2. Physiological pathways and metabolism

Numerous enzymes and transporters are involved in the carnosine metabolism, demonstrating that the carnosine system is subject to a complex homeostatic regulation. As carnosine and anserine are highly present in skeletal muscles of mammals and fish, omnivorous subjects have a daily consumption of these dipeptides with the ingestion of meat and fish. Interestingly, HCDs are the only source of beta-alanine in an omnivorous diet, while L-histidine is present in most protein-rich foods, such as meat and fish, but also dairy and grain products.

The main pathways involved in the regulation of tissue carnosine levels are synthesis from its precursor amino acids, which is catalyzed by **carnosine synthase** (CARNS), and hydrolysis into its constituents by carnosinases (CN). Carnosine synthesis mainly takes place in skeletal muscles, whereas 2 forms of carnosinase exists in humans: **serum carnosinase** (CN1) and **tissue carnosinase** (CN2). CN1 is highly active in humans, meaning that only negligible levels of carnosine are detectable in the blood, whereas CN2 is present in many tissues such as liver, kidney and small intestine (Lenney *et al.*, 1985). These enzymes will be further described in the following sections.

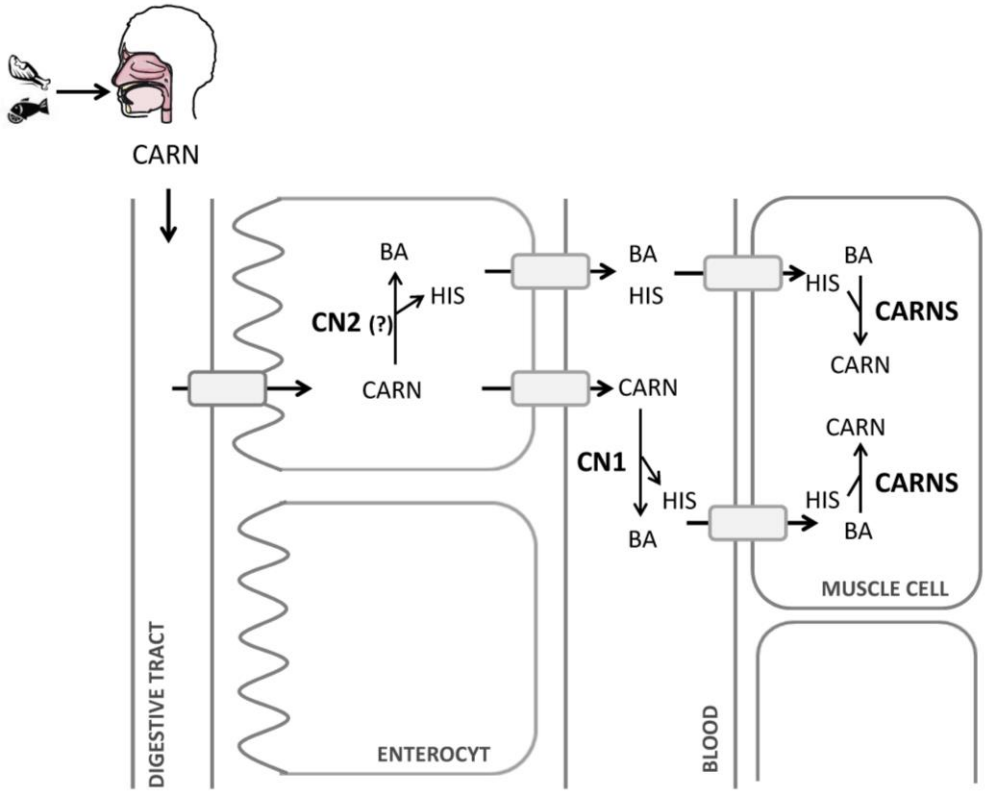

Figure 3: Possible pathways of intestinal absorption of carnosine. BA: beta-alanine. CARN: carnosine; CARNS: carnosine synthase; CN1: serum carnosinase; CN2: tissue carnosinase; HIS: histidine.

Thus, following **HCD ingestion** through meat or fish, the presence of CN2 in the enterocytes hydrolyzes carnosine into beta-alanine and L-histidine before reaching the blood stream. However, since the CN2 activity in rather low (Sadikali *et al.*, 1975), it is very likely that part of the ingested carnosine reaches the blood stream intactly, whereupon it is rapidly hydrolyzed in plasma due to the high activity of CN1 (Fig 3). The constituent amino acids beta-alanine and L-histidine are then transported in the muscle cells by their respective amino acid transporters, where CARNS is present to synthesize carnosine (Bakardjiev & Bauer, 1994; Drozak *et al.*, 2010). The metabolic pathways in which beta-alanine and L-histidine are incorporated as well as the enzymes and transporters for these amino acids will be discussed in detail in section 4.

As mentioned, carnosine is the only HCD found in human muscle. However, in rodents, as in most animals, the methylated form, in this case anserine, is more abundant than the non-methylated carnosine. There are two possible pathways for the synthesis of the methylated carnosine analog anserine. Firstly, **carnosine-N-methyltransferase** (CMT) catalyzes the transfer of a methyl group on carnosine to form anserine and secondly, direct enzymatic condensation of beta-alanine with methylhistidine can be catalyzed by CARNS. Up to now, it is not yet fully understood which pathway is the main source of methylated analogs. Recent findings consistently found methyl-L-histidine to be a poorer substrate for CARNS than nonmethylated L-histidine (Drozak *et al.*, 2010). Furthermore, using radiolabeled beta-alanine in primary culture myocytes, carnosine synthesis was shown to be the first and major pathway, followed by methylation to form anserine (Bauer & Schulz, 1994). Together, these findings suggest that CMT is the major enzyme responsible for anserine synthesis. Interestingly, Drozak et al. (2015) recently molecularly identified UPF0586 protein C9orf41 as the mammalian carnosine-N-methyltransferase, responsible for anserine formation in rat muscle. Moreover, the authors demonstrated a low CMT mRNA expression in human muscle, confirming the absence of anserine in human myocytes, but an unexpectedly high expression of UPF0586 mRNA was found in human kidney, suggesting that the enzyme may be active, leading to the accumulation of anserine in renal tissue (Fig 4). Indeed, the presence of anserine in human renal tissue was very recently confirmed by Peters et al. (2015).

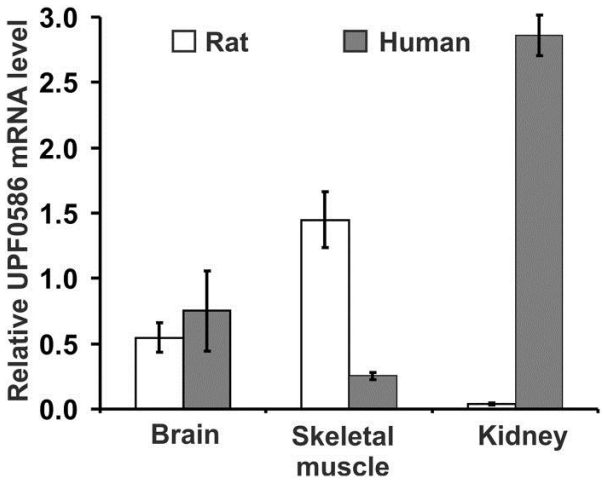

Figure 4: Relative mRNA expression for rat and human UPF0586 protein determined in brain, skeletal muscle and kidney tissues, as demonstrated by Drozak et al. (2015)

One decade ago, Harris and coworkers (2006) demonstrated for the first time that **chronic oral beta-alanine supplementation** is able to enhance muscle carnosine levels and thus disturb carnosine homeostasis, thereby marking beta-alanine as the **rate-limiting precursor** for carnosine synthesis. As it was subsequently shown that elevated muscle carnosine levels lead to increased performance in various high-intensity exercise models (Hill *et al.*, 2007), this opened up perspectives for the carnosine research topic. The enzymes involved in beta-alanine metabolism will be discussed in section 4. Failure to maintain carnosine homeostasis upon beta-alanine supplementation and the course of a beta-alanine supplementation protocol will be further elucidated in section 5.

2.3. Biochemical properties of carnosine

Although carnosine is one of the most abundant metabolites in skeletal muscles, it is not involved in any energy delivering pathways. However, several physiological properties of carnosine are relevant to muscular function and homeostatic processes, such as pH buffering, increasing Ca^{2+} sensitivity, antioxidant capacity and inhibiting protein glycation. Below, these functions will be further clarified.

2.3.1. pH buffer

The role of carnosine as a physiologically relevant pH buffer was the first function of the dipeptide to be discovered. During high-intensity exercise, anaerobic glycolysis is the major energy providing pathway, resulting in the intramyocellular production of lactic

acid, which dissociates to lactate and protons (H^+). Due to the accumulation of H^+ ions, a fall in the pH from 7.1 in rest up to 6.3 during exercise occurs, disturbing intramuscular acid-base homeostasis. To counteract or delay this process, different metabolites are able to accept H^+, thereby functioning as proton buffers to restore homeostasis. The most important intramuscular proton buffers are inorganic phosphate, bicarbonate and proteins and dipeptides. The imidazole ring of L-histidine is capable to accept one proton, suggesting that free L-histidine can act as a proton buffer. However, the pKa value (acid dissociation constant) of L-histidine is 6.1, which is close to but not exactly in the physiological pH range of contracting and fatigued myocytes. Thus, in actual practice, the contribution of free L-histidine to the buffering capacity is rather limited. Yet, by combining L-histidine with beta-alanine, the pKa of the imidazole ring alters to 6.83, making carnosine an ideal intracellular buffer, although the imidazole of L-histidine regulates the buffering activity of carnosine.

The relative buffering capacity in the human vastus lateralis muscle was determined to be at least 4.5 and 9.4% in type I and type II fibers, respectively (Mannion *et al.*, 1995). Since carnosine is a mobile buffer, freely dissolved in the cytoplasma of myocytes, its contribution to pH homeostasis is probably even greater than previously calculated (Boldyrev *et al.*, 2013). Moreover, increasing muscle carnosine concentrations by beta-alanine supplementation enhances the buffering capacity of muscles, demonstrated by an attenuated decline in blood pH during a 6-minute high-intensity cycling exercise test (Baguet *et al.*, 2010*b*).

2.3.2. Ca^{2+}-regulator

During the excitation-contraction coupling in skeletal muscle cells, release of calcium from the sarcoplasmic reticulum and binding of calcium to troponin are key steps to induce contraction of myocytes. Both in rat and in human skinned muscle fibers (Dutka & Lamb, 2004; Dutka *et al.*, 2012), raised cytoplasmic carnosine concentrations increase Ca^{2+} sensitivity of the contractile apparatus in a concentration-dependent manner. In addition, these observations were also replicated in whole incubated skeletal muscles of mice (Everaert *et al.*, 2013*b*). Up to now, enhanced calcium handling by carnosine loading could only be demonstrated by in vitro studies or by in vivo beta-alanine administration

and ex vivo effect measurements. Experiments using in vivo models are necessary to further fortify the role of enhanced carnosine levels for increasing calcium sensitivity in muscle fibers.

Recently, the two functions of carnosine as described above, were united in one concept, named the **carnosine shuttle** (Fig 5). This concept is based on recent findings in cardiac myocytes demonstrated by Swietach et al. (2013, 2014) and holds that carnosine can act as a diffusible Ca^{2+}/H^+ exchanger. On the one hand, H^+ accumulation at the sarcomere site occurs as a result of anaerobic glycolysis. These ions need to be transported to the sarcolemma to drive trans-sarcolemmal H^+ export. On the other hand, Ca^{2+} needs to diffuse in the opposite direction i.e. from the sarcoplasmic reticulum to the sarcomeres, to promote cross-bridge formation. As both H^+ and Ca^{2+} can competitively bind to carnosine and carnosine is a mobile buffer, it can improve calcium delivery to and proton removal from the sarcomere site.

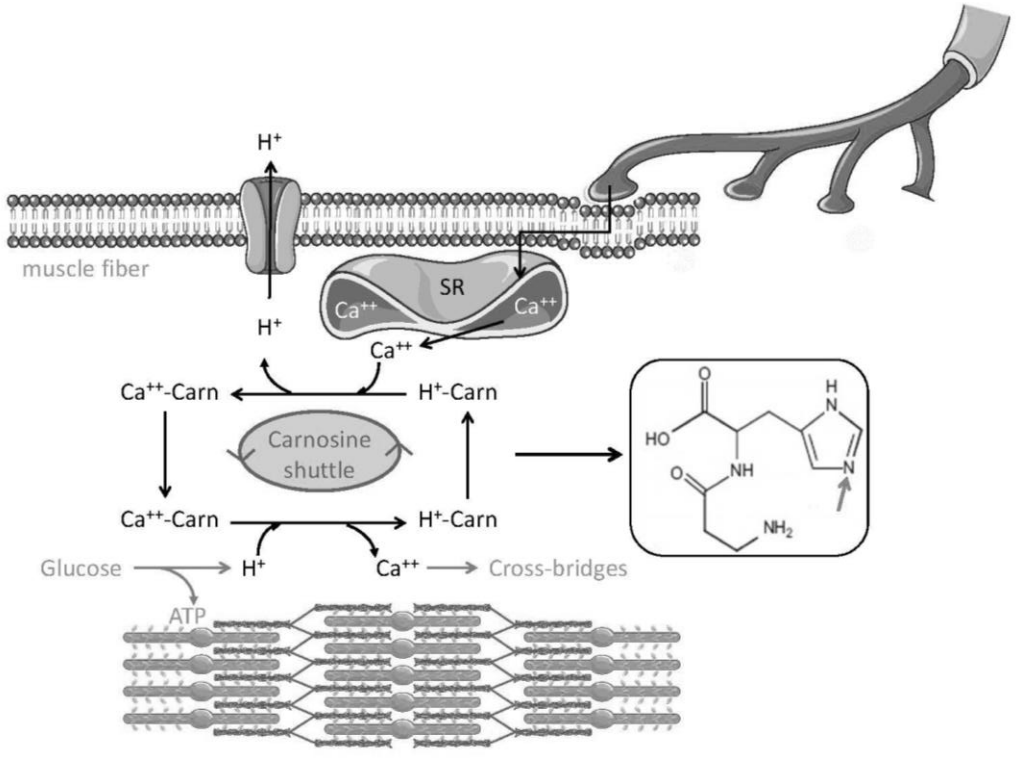

Figure 5: Current hypothesis on the ergogenic mechanism of carnosine in skeletal muscle, based on the findings of Swietach et al. (2013, 2014). It poses that carnosine can act as a shuttle by transporting both Ca^{2+} and H^+ between sarcomere region and the subsarcolemmal region. Molecular structure of carnosine is shown with indication (arrow) to the competitive binding site of H^+ and Ca^{2+} (reviewed by Blancquaert et al. (2015)). SR: sarcoplasmic reticulum

2.3.3. Anti-oxidant and anti-glycation

Reactive oxygen species (ROS) are a naturally occurring byproduct of mitochondrial respiration and have an important role in cell signaling and homeostasis. However, when ROS levels reach high concentrations, damage to cell structures such as DNA and proteins may occur, which is known as oxidative stress. To protect from this stress, our body possesses both enzymatic (such as catalase and superoxide dismutase) and non-enzymatic (vitamins) anti-oxidants, capable of disarming ROS. Carnosine is demonstrated to be a non-enzymatic natural antioxidant as its imidazole moiety can interact with singlet oxygen and scanvenge peroxyl radicals and superoxide radicals (Kohen & Yamamoto, 1988). Furthermore, in vitro studies have shown that carnosine can inhibit glycation and protein-protein cross-linking, thereby interfering the formation of advanced glycation end-products (Hipkiss, 2005). Based on these properties, carnosine has been suggested to be a potential therapeutic compound in a number of pathologies.

3. Set point of carnosine

As mentioned, carnosine is one of the most abundant molecules in skeletal muscle. In the following section, different factors determining the carnosine set point and normal range as well as the importance of this high set point is discussed.

3.1. Homeostatic set point and normal range

Carnosine has a **high homeostatic set point** in human muscles. Based on a database of proton MRS carnosine measurements in calf muscles that were collected over the past years in our lab, it can be concluded that the set point amounts to 4.11 mM in soleus muscle and 6.96 mM in gastrocnemius muscle. This database includes both males and females and subjects of all age classes (ranging from 8 to 83 years). Repeated measurements of muscle carnosine levels over time in the same people displays only small fluctuations, demonstrating a low intra-individual variation and a **high stability of the carnosine set point.** For soleus and gastrocnemius muscle, variation coefficients over a 3 month period were calculated to be 9% and 15%, respectively (Baguet *et al.*, 2009). The methodological variation of the MRS has been previously determined to be 4.3% for soleus and 7.6% for gastrocnemius muscle, when subjects are measured twice on the same day (Ozdemir *et al.*, 2007). Thus the biological variation of muscle carnosine content over a 3 month period is as low as ~6% (Baguet *et al.*, 2009). This variation coefficient resembles the **normal range** of the carnosine set point and illustrates that carnosine homeostatis is a **dynamic process**, subject to feedback processes to keep carnosine between certain fixed limits (Fig 6).

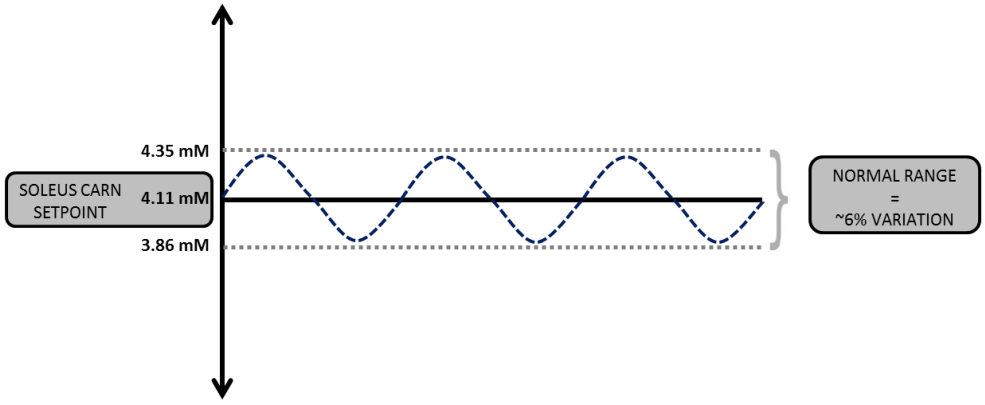

Figure 6: Graphical presentation of proposed dynamic homeostatic regulation over time of the carnosine set point in soleus muscle

3.2. Inter-individual and inter-fiber differences in the carnosine set point

Although the carnosine set point only minimally fluctuates over time (low intra-individual variability), there is a **large inter-individual variation in this set point** in human skeletal muscle. Three- to fourfold differences have been demonstrated between the lowest and the highest reported levels in humans. The concentration of muscle carnosine in a set of humans follows a Gaussian distribution, with variation coefficients of 27% in soleus and gastrocnemius muscle (Derave *et al.*, 2010). A histogram of the current data from the carnosine measurements of our database, showing the Gaussian distribution, is depicted in figure 7. Some determinants are known to influence this set point, such as age, gender, diet and muscle fiber type composition (Fig 8).

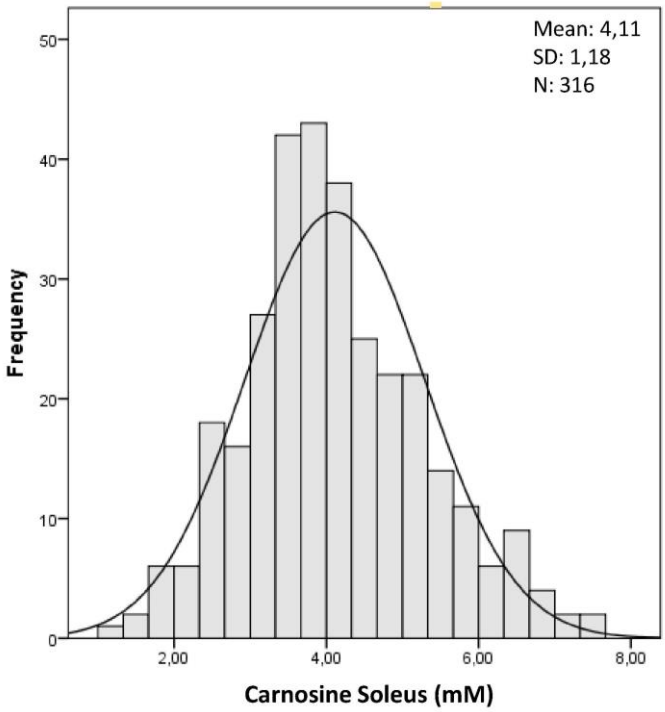

Figure 7: Gaussian distribution of the carnosine content of the soleus muscle in a general population (both males and females, age 8-83years, n=316)

In general, the muscle carnosine set point is **higher in men compared to women**. In our database, the mean soleus carnosine concentration is 3.68 mM in women and 4.47 mM in men. This is in accordance with the data of Mannion and colleagues (1995) who showed that men have approximately 20-25% higher carnosine levels in the vastus lateralis muscle compared to age-matched women. Baguet and colleagues (2012) measured muscle carnosine levels cross-sectionally on different timepoints during the human

lifespan to obtain a clear understanding of the impact of puberty and **aging** on human muscle carnosine concentration. They found that the carnosine set point is upregulated (reaching a higher homeostatic set point) during puberty in males, but not in females. Alternatively, in both males and females, an age-related downregulation (reaching a lower homeostatic set point) of the carnosine set point was found mainly during early adulthood, but not from adulthood to elderly.

As mentioned, HCDs are present in meat and fish and omnivores thus have a daily dietary ingestion of these metabolites. Interesting to note is that there is no correlation between dietary beta-alanine ingestion (within the normal range of 200–400 mg per day (Everaert *et al.*, 2011)) and baseline muscle carnosine concentrations (Baguet *et al.*, 2009; Stellingwerff *et al.*, 2012*a*), suggesting that normal variations in the **dietary HCD** intake are not greatly affecting muscle carnosine homeostasis. Yet, cross-sectional data on long-term vegetarians, who have a complete restriction of dietary HCDs and thus also beta-alanine (Table 1), suggests that they have a somewhat lower muscle carnosine set point compared to omnivorous subjects (Harris *et al.*, 2007; Everaert *et al.*, 2011). However, the currently available cross-sectional data are not very convincing. The study of Harris et al. (2007) included only 6 vegetarians and was not gender- and age-matched, although these are important determinants of the carnosine set point, as described above. The study of Everaert et al. (2011) only found significant lower muscle carnosine content in the gastrocnemius of 12 vegetarians compared to omnivores, while no significants effects were noted in soleus and tibialis anterior muscles. Human long-term intervention studies investigating the effects of transiently switching omnivores onto a vegetarian diet are necessary to confirm these cross-sectional findings.

Table 1: Overview of the amount of beta-alanine, carnosine and histidine in omnivorous and vegetarian diets. Beta-alanine is only present in the form of carnosine or anserine (1g of carnosine contains 400mg of beta-alanine), while histidine is present is all protein-rich foods.

	Beta-alanine	**Carnosine or anserine**	**Histidine**
Omnivorous diet	300-600mg[1]	800-1600mg	1.5-2.5g[2]
Vegetarian diet	/	/	1.5-2g ?[3]

1 Everaert et al (2011), Saunders et al (unpublished data)
2 Reeves et al (1977), Okubo et al (2005)
3 No data available. Because fish and meat contain the highest histidine to protein ratio, dietary histidine intake might be somewhat lower in vegetarians

A last determinant which is known to influence the muscle carnosine set point is muscle **fiber type composition**. Fast-twitch muscle fibers (type II fibers) are shown to contain twice as much carnosine compared to slow-twitch muscle fibers (type I fibers) (Harris *et al.*, 1998; Hill *et al.*, 2007). Accordingly, carnosine homeostatic set point is significantly higher in the fast-twitch gastrocnemius than the more slow-twitch tibialis anterior and soleus (Baguet *et al.*, 2009; Derave *et al.*, 2010). In our database, set point of gastrocnemius amounts to 6.76 mM, which is 60% higher than the homeostatic set point is soleus (4.06 mM).

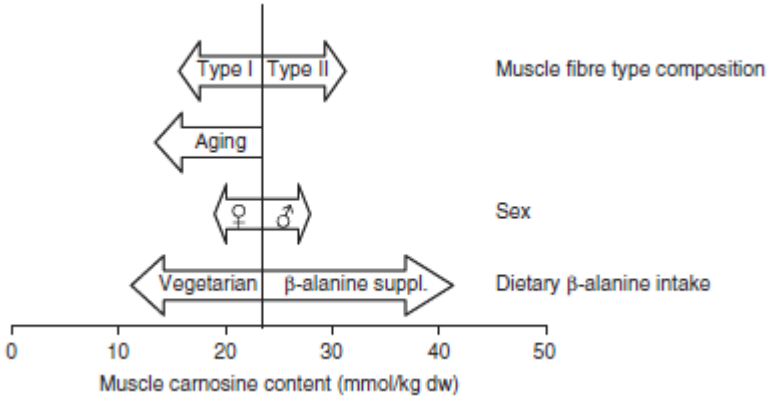

Figure 8: Putative determinants of the human muscle carnosine content, as depicted by Derave et al. (2010). DW: dry weight; suppl: supplementation

Aims: Age, gender, diet and muscle fiber type composition are generally shown to be the main determinants of the carnosine set point. Evidence for the determinant diet is based on cross-sectional data demonstrating somewhat lower muscle carnosine levels in long-term vegetarians compared to omnivores. Similarly, cross-sectional studies suggested that the homeostasis of plasma and muscle creatine and carnitine, two other compounds that are almost solely found in tissues of animals (carninutrients), is negatively affected by long-term vegetarianism (Delanghe *et al.*, 1989; Burke *et al.*, 2003; Stephens *et al.*, 2011). The effect of switching omnivores onto a long-term vegetarian diet is until now unexplored. Therefore, an aim of this PhD thesis is to explore the effect of long-term vegetarianism in previously omnivorous subjects on the homeostasis of different carninutrients such as carnosine, creatine and carnitine.

3.3. Why do we need a high muscle carnosine set point?

As already described above, several physiological functions are ascribed to carnosine. Based on its biochemical properties, it can be stated that the carnosine system has evolved as a pluripotent solution to a number of homeostatic challenges.

When carnosine is discussed or reviewed, beta-alanine commonly draws most attention as it is mainly regulating the carnosine synthesis. However, most **bioactive functions of carnosine relate to L-histidine** and more specifically its imidazole moiety (Fig 9). Based on this, it can be questioned what the advantages of a L-histidine containing dipeptide are, compared to free L-histidine. The reason for the development of the carnosine system probably relates to improvement of the functional activities (i.e pKA values of carnosine is closer to the physiological range than the pKA of free L-histidine) and guaranteeing a stable tissue concentration. L-histidine is, next to incorporation in carnosine, also involved in many other pathways such as protein synthesis and histamine formation. As carnosine is mainly stored in muscle, and carnosinase is considered not to be active in muscle cells, carnosine and thus also L-histidine content is highly stable.

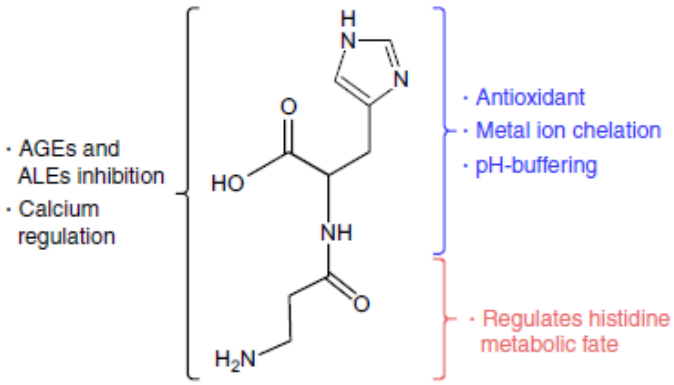

Figure 9: Structure-activity relationship of carnosine (Boldyrev *et al.*, 2013)

3.4. Tissue differences

With muscle concentrations as high as in the millimolar range, 99% of the total body carnosine in an organism is found in **skeletal muscle tissue**. Accordingly, the highest rate of CARNS activity is found in mammalian skeletal muscle tissue (Abe, 2000) and the enzyme was shown to be highly expressed on mRNA level in human skeletal muscle (Everaert *et al.*, 2013*a*). Next to a high concentration in muscle, carnosine is present in

excitable neuronal tissues and different parts of the brain, with the highest content found in the olfactory bulb, which displays very high CARNS expression (Horinishi *et al.*, 1978). In other brain structures, however, homocarnosine rather than carnosine is the main dipeptide.

Carnosine along with mRNA expression of CARNS was also recently detected in liver, kidney, retina and spleen rat tissues (Kamal *et al.*, 2009; Mong *et al.*, 2011; Pfister *et al.*, 2011; Riedl *et al.*, 2011), but in concentrations 10- to 1,000-fold lower than in muscle (Boldyrev *et al.*, 2013) (Table 2). Mammalian cardiac muscle is also shown to have only a relatively low concentration of carnosine (~0.1 mmol/kg wet tissue) (O'Dowd *et al.*, 1988). However, the total content of carnosine derivatives (acetylated carnosine, anserine and homocarnosine) is high and in the order of 2–10 mmol/kg wet tissue.

Carnosine is **undetectable in fasted human plasma** as it is readily degraded by the very active serum carnosinase enzymes (CN1). In contrast to humans, CN1 is only present in the kidneys and not in the circulation of rodents (Peters *et al.*, 2012). Interestingly, a recent study demonstrated the presence of carnosine and anserine in human renal tissue (Peters *et al.*, 2015). Moreover, the authors demonstrated mRNA expression of CARNS, CNDP1 (gene encoding CN1) and taurine transporter (=transporter for beta-alanine) in distinct compartments within the nephron, indicating that the kidney has an intrinsic capacity to metabolize carnosine (Table 2). Literature on profiling of carnosine content and CARNS mRNA expression in human tissues other than skeletal muscle and kidney is currently lacking due to the inconvenience to sample these tissues.

Carnosinase is also present in tissues as non-specific dipeptidase or tissue carnosinase (CN2, encoded by the gene CNDP2), which has been demonstrated in kidney, liver, spleen and small intestine (Lenney *et al.*, 1985). Moreover, mRNA expression was also found in skeletal muscle (Lenney *et al.*, 1985; Everaert *et al.*, 2013*a*) (Table 2). Yet, the catalytical rate of CN2 in tissues is markedly lower than the catalytical rate of CN1 in serum (Pandya *et al.*, 2011). Furthermore, CN2 was shown to have a broader substrate specificity than CN1 and the optimum pH for CN2 to degrade carnosine is 9.5, which is not in the physiological pH range (Teufel *et al.*, 2003). It thus remains doubtful whether CN2 can degrade carnosine in vivo. It is shown however that Dug1, the yeast orthologue of

mammalian CN2, is a highly specific Cysteine-Glycine (Cys-Gly) dipeptidase. Furthermore, CNDP2 has been shown to have Cys-Gly dipeptidase activity in vitro and moreover, CNDP2 can complement the defective utilization of Cys-Gly of a Dug1-deficient mutant (Kaur *et al.*, 2009).

Table 2: Current belief on the presence of the most important enzymes in the metabolism of carnosine in several tissues of both rodents and humans

RODENT	Blood	Skeletal muscle & heart	Brain/CNS	Other organs	
CARNS	-	++	++	+ Kidney Liver Retina	++ Spleen
CN1	-	-	-	+ Small intestine	++ Kidney
CN2	-	+	+	+ Liver	++ Small intestine Kidney
HUMAN	**Blood**	**Skeletal muscle & heart**	**Brain/CNS**	**Other organs**	
CARNS	-	++	++	+ Kidney	
CN1	++	-	+	+ Liver Kidney	
CN2	-	+	+	+ Lung Spleen Liver	++ Kidney Small intestine

- Absent, + little present, ++ abundantly present. CN1: serum carnosinase; CN2: tissue carnosinase, CARNS: carnosine synthase; CNS: central nervous system. Based on information from the online databases biogps and gtexportal.

4. Potential effectors in the control of carnosine homeostasis

The regulation of body and more specifically muscle carnosine homeostasis is until now poorly understood and involves a complex set of enzymes and transporters. The availability of the rate-limiting precursor beta-alanine and the enzyme catalyzing the dipeptide synthesis (CARNS) are believed to be key steps. Several of the enzymes and transporters which regulate carnosine homeostasis have only recently been molecularly identified which contributes to a better understanding of the regulation of carnosine homeostasis. Figure 10 gives an overview of the several steps and their respective enzymes and transporters that are possibly involved in the carnosine metabolism. These steps include: **beta-alanine transport** (TauT, PAT1), beta-alanine availability regulated by **beta-alanine synthesis** (GADL1 and uracil degradation) on the one hand, and **beta-alanine degradation** (GABA-T and AGXT2) on the other hand, **histidine transport** (PHT1, PHT2), **histidine degradation** (HDC) and **carnosine synthesis** (CARNS) and **degradation** (CN1 and CN2).

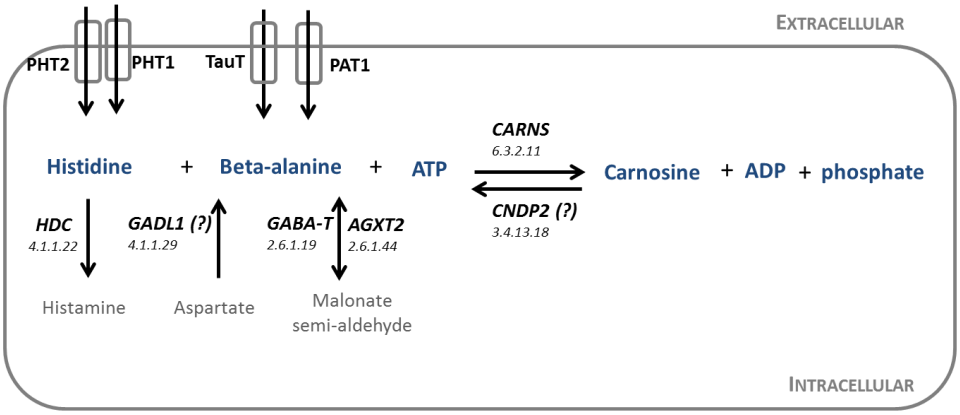

Figure 10: Enzymes (with their respective EC number) and transporters possibly involved in the carnosine metabolism

This section will discuss the presence of the carnosine-related enzymes and transporters in different parts of the body to demonstrate how body carnosine homeostasis is regulated. For each of the enzymes and transporters, the presence in muscle will be discussed more in detail, as the following sections will focus specifically on regulation of muscle carnosine homeostasis.

4.1. Intracellular beta-alanine availability

Beta-alanine is a rather unusual amino acid. It is not used in the biosynthesis of proteins (nonproteinogenic) but is a naturally occurring free beta-amino acid. Below, the most

important proteins that play a role in beta-alanine transport, namely TauT and PAT1, are defined. Moreover, pathways for beta-alanine synthesis, including uracil degradation and aspartate decarboxylation by the GADL1 enzyme, and pathways for beta-alanine degradation (GABA-T and AGXT2) are clarified.

4.1.1. Beta-alanine transport

The first and crucial step for intramyocellular carnosine synthesis is uptake of beta-alanine from the plasma into the muscle cells. The intramyocellular beta-alanine concentration is very low (< 10µmol/kg wet weight) and Harris et al. (2006) demonstrated that the synthesis of carnosine is limited by the availability of beta-alanine, making this step indispensable in the muscle carnosine synthesis process (Artioli *et al.*, 2010). Three transporters capable of transferring beta-alanine across the sarcolemma are known, namely PAT1, Taurine transporter (TauT) and $ATB^{0,+}$.

PAT1 (proton-coupled amino acid transporter 1) is a protein, encoded by the SLC36A1 gene in humans (Metzner *et al.*, 2006). PAT1 is a proton driven, pH-dependent transporter characterized as a high-capacity, low-affinity transporter. The substrate and the proton are translocated at a coupling stoichiometry of 1:1 (Boll *et al.*, 2002). PAT1 is able to transport both beta-alanine and taurine (Anderson *et al.*, 2009). TauT is encoded by the SLC6A6 gene in humans (Anderson *et al.*, 2009). Contrary to PAT1, TauT is a high-affinity, low-capacity transporter and is Na^+- and Cl^- dependent ($2Na^+$: $1Cl^-$: 1 taurine) (Bakardjiev & Bauer, 1994; Han *et al.*, 2006). Similar to PAT1, both beta-alanine and taurine are suitable substrates for this transporter (Anderson *et al.*, 2009). As both transporters are able to use beta-alanine and taurine as substrates, it can be suggested that these substrates competitively bind to the transporters for transsarcolemmal uptake. The evidence that plasma taurine levels are elevated after acute beta-alanine supplementation (Harris *et al.*, 2006) subscribes this notion. $ATB^{0,1}$ (encoded by the human SLC6A14 gene) is a transporter with similar characteristics as TauT, although it only accepts beta-alanine but not taurine (Anderson *et al.*, 2009).

Expression of the PAT1 and TauT carrier proteins was already detected in the intestine, brain and liver (Metzner *et al.*, 2006) and all three transporters are shown to be expressed in human duodenum and ileum (Anderson *et al.*, 2009). Moreover, expression

of TauT and PAT1 has been demonstrated in skeletal muscle of rodents (Pierno *et al.*, 2012) and humans (Drummond *et al.*, 2010, 2011). Recently, Everaert et al. (2013*a*) confirmed the mRNA expression of TauT and PAT1 in skeletal muscle of both adult mice and humans, whereas no mRNA expression of $ATB^{0,1}$ was found in both species, suggesting this transporter is less or not important in the regulation of muscle carnosine homeostasis.

4.1.2. Beta-alanine synthesis and degradation

Until now, the only known endogenous pathway to synthesize beta-alanine in humans is the three-step uracil degradation in liver (Matthews *et al.*, 1987; Traut, 2000). Furthermore, Liu et al. (2012*a*) demonstrated that insects have a more straightforward enzymatic pathway to produce beta-alanine, namely through aspartate decarboxylation catalyzed by the aspartate 1-decarboxylase enzyme (ADC). In mammals, ADC is not found, but it has been proposed that there is a specific enzyme in mammalian muscle, namely glutamate decarboxylase-like protein 1 (GADL1; EC 4.1.1.29), directly synthesizing beta-alanine from aspartate by a similar decarboxylation reaction as occurs in insects (Liu *et al.*, 2012*a*). Despite its name, GADL1 has no detectable glutamate decarboxylase activity. GADL1 mRNA expression was found in mouse and cattle skeletal muscles and also in mouse kidneys. Western blot analysis verified the presence of GADL1 in mouse muscles and kidneys. Until now, mRNA expression of GADL1 in human muscle cells is uninvestigated. Using recombinant DNA technology, beta-alanine-producing activity of GADL1 could not yet be detected in the supernatant of tissue protein extracts. Yet, the potential role of GADL1 in beta-alanine synthesis cannot be excluded (Liu *et al.*, 2012*a*). Interestingly, this enzyme was also suggested to be involved in taurine biosynthesis by cysteine sulfinic acid decarboxylase activity, which might indicate that beta-alanine and taurine biosynthesis may involve the same enzyme.

A third pathway related to beta-alanine metabolism encloses the transaminase enzymes, a group of enzymes that may be involved in both beta-alanine synthesis and degradation. As the name implies, transamination refers to the exchange of an aminegroup (NH_2) of one molecule with the ketogroup (=O) of another molecule (Fig 11). Transamination reactions are known to be readily reversible. The direction is determined by which of the reactants are in excess. Transaminases are pyridoxal-5'-phosphate (PLP) dependent

enzymes, indicating that PLP, the active form of vitamin B6, is a necessary co-factor to complete the reactions.

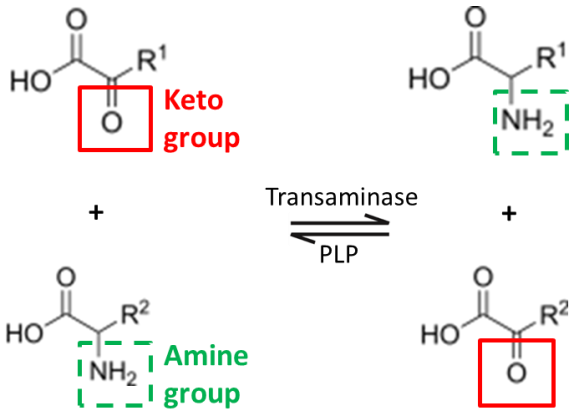

Figure 11. Schematic overview of transamination reactions

Pihl & Fritzson (1955) reported that more than 90% of the injected C^{14}-labelled beta-alanine in rats was recovered in the expired CO_2 in 5h, suggesting that beta-alanine can be metabolized elsewhere, most probably as a carbon source for energy provision through oxidation. Indeed, the amine group of beta-alanine can be removed through a transamination resulting in the formation of the keto-acid malonate semi-aldehyde (MSA), which can subsequently enter the citric acid cycle and provide energy. Mostly based on enzymatic assays in cell extracts, two mitochondrial enzymes are known to catalyze this reaction: 4-aminobutyrate-2-oxoglutarate transaminase (EC 2.6.1.19; also known as GABA-T or β-alanine-2-oxoglutarate transaminase) (Ito *et al.*, 2001) and alanine-glyoxylate transaminase (EC 2.6.1.44; also known as AGXT2 or β-alanine-pyruvate transaminase) (Hayaishi *et al.*, 1961; Rodionov *et al.*, 2014) (Fig 12). Until now, GABA-T and AGXT2 are, to our knowledge, the only known mammalian enzymes that can transaminate beta-alanine.

Jeon et al. (2000) investigated the tissue distribution of GABA-T mRNA and found highest expression in liver, brain, pancreas and kidney, whereas low expression was found in heart and reproductive organs, and only trace amounts of expression or no clear signal in other tissues, for example skeletal muscle and lung. Yet, Everaert et al. (2013*a*) were able to identify GABA-T mRNA expression in mice skeletal muscle. Alike GABA-T, AGXT2 is primarily expressed in kidney and liver and to a much smaller extent in skeletal muscle (Lee *et al.*, 1999; Rodionov *et al.*, 2014).

Transaminases are known to work in two directions, suggesting that GABA-T and AGXT2 transaminases are also capable to synthesize beta-alanine from malonate semi-aldehyde. Until now, this possible beta-alanine synthesizing pathway upon malonate semi-aldehyde administration is poorly investigated in mammals. Very recently, Wilson and colleagues presented a conference poster (2016) demonstrating that, when perfusing rat liver with 3-hydroxypropionate (a precursor of MSA), beta-alanine was synthesized. This is the first *in situ* evidence for the beta-alanine synthesizing capacity of beta-alanine transaminases GABA-T and/or AGXT2.

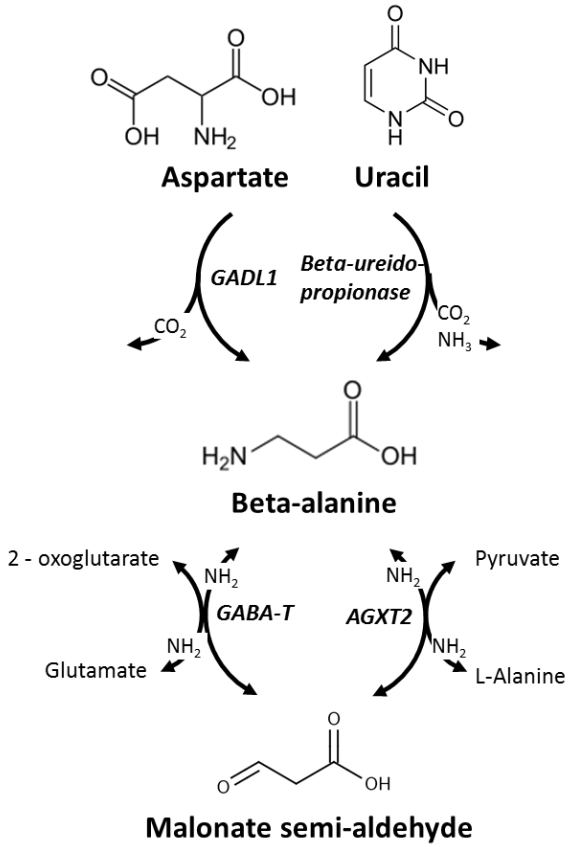

Figure 12: Reaction scheme for the beta-alanine synthesis and degradation pathways. Uracil degradation is a three-step pathway of which only the last enzyme is mentioned in this figure.

4.2. Intracellular L-histidine availability

Next to beta-alanine, carnosine synthesis also requires the availability of the second precursor of the dipeptide, namely L-histidine. L-histidine is a proteinogenic alfa-amino acid. It is generally believed that the magnitude of carnosine synthesis is more dependent upon the availability of beta-alanine compared to L-histidine, although the effect of elevated histidine availability on muscle carnosine loading is until now never explored.

The histidine-related transporters (PHT1 and PHT2) and enzymes (HDC) are discussed below.

4.2.1. L-histidine transport

Two proteins, PHT1 and PHT2, are known to transport L-histidine. PHT1 and PHT2 are members of the proton-coupled oligopeptide transporters (POT-family or SLC15). The members of the POT family are PEPT1 and PEPT2 (oligopeptide transporter 1 and 2) and PHT1 and PHT2 (peptide/histidine transporter 1 and 2). All of these POTs are able to transfer di- and tripeptides (including carnosine and its methylated analogs) across biological membranes. PHT1 and PHT2 differ from the PEPTs as PHTs also recognize, in addition to di/tripeptides, the amino acid L-histidine as a substrate (Daniel, 2004). Everaert et al. (2013*a*) recently demonstrated the mRNA expression of PHT1 and PHT2 in mouse and human skeletal muscle samples, but it is currently unclear what their physiological function is in muscle and whether inward or outward transport is the main direction (Boldyrev *et al.*, 2013).

4.2.2. L-histidine synthesis and degradation

L-histidine is a semi-essential and proteinogenic amino acid. Histidine is categorized as a semi-essential amino acid because it can not be synthesized *de novo* in mammals and is only indispensable in certain populations or situations, in which ingestion of histidine through the diet is thus essential. Generally, children need to consume L-histidine in their diet, while this is no longer essential in adults (Stifel & Herman, 1972). L-histidine biosynthesis is an ancient metabolic pathway present in bacteria and plants but not or only minimally present in mammals. Under normal physiological conditions, the histidine concentration in biological tissues is sufficiently present.

Furthermore, histidine decarboxylase (HDC, EC: 4.1.1.22) catalyzes the formation of histamine from L-histidine. Histamine is an organic compound involved in a series of physiological processes, such as local immune responses and inflammatory responses. Interestingly, also some beneficial exercise-related roles are ascribed to histamine such as the vasodilating effect of histamine on arterioles in (post-)exercise hyperaemia (Jones, 2016). HDC is shown to be expressed in both mouse (Everaert *et al.*, 2013*a*) and human skeletal muscle and is upregulated following exercise (Niijima-Yaoita *et al.*, 2012; Romero

et al., 2016), suggesting that histidine might be degraded in muscle cells, especially during exercise.

4.3. Carnosine metabolism

4.3.1. Carnosine synthesis

In the 1950s, the biochemical properties of the carnosine synthesis reaction were established by in vitro experiments on partially purified enzyme preparations of chicken muscle, and the enzyme was called carnosine synthase (CARNS; 6.3.2.11). It became clear that, in addition to the constituent amino acids beta-alanine and L-histidine, Mg^{2+} and ATP are required to synthesize carnosine, making carnosine synthesis an energy-consuming molecular process (Fig 13) (Kalyankar & Meister, 1959).

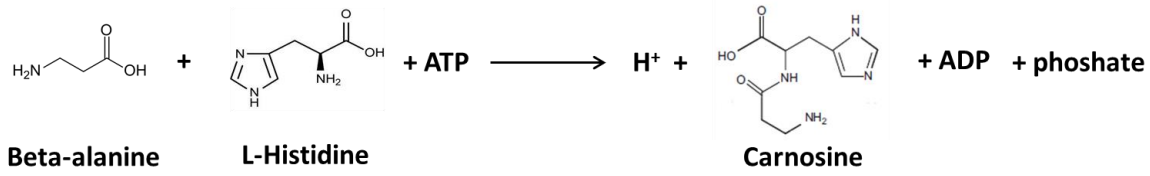

Figure 13: Carnosine formation scheme

The gene encoding this enzyme has been molecularly identified by Jakub Drozak and coworkers (2010). The gene encoding carnosine synthase is ATPGD1, a member of the ATP-grasp superfamily. As mentioned, CARNS is a cytosolic enzyme and is mainly present in skeletal and heart muscle and certain brain regions. Next to carnosine synthesis, CARNS is also the responsible enzyme for homocarnosine synthesis from GABA and L-histidine.

4.3.2. Carnosine degradation

As beta-alanine is a rare, non-proteinogenic amino acid, carnosine and related compounds have a low affinity towards hydrolysis by regular (di)peptidases. Therefore, carnosine is characterized by its own, separately regulated hydrolytic enzymes, named carnosinases. Two forms of carnosinase have been molecularly identified as CN1 or serum carnosinase (3.4.13.20) and CN2 or tissue carnosinase (3.4.13.18). The genes of these carnosinases are CNDP1 and CNDP2, respectively.

CNDP1 is expressed in human liver and the carnosinase enzyme is subsequently secreted in the circulation. Thus, serum carnosinase activity is high in the adult human, leading to

almost undetectable levels of circulating carnosine in the postabsorptive state (Gardner *et al.*, 1991). The activity of CN1 varies greatly between individuals and mostly reaches higher levels in females compared to males (Peters *et al.*, 2010). CN1 activity is much lower for anserine and homocarnosine compared to carnosine. In rodents, CNDP1 is exclusively expressed in the kidney (Teufel *et al.*, 2003) and CN1 is, in contrast to humans, absent in the circulation. CNDP2 is widely distributed in central and peripheral human tissues such as liver, kidney, spleen, small intestine and even muscle (Lenney *et al.*, 1985; Everaert *et al.*, 2013*a*). It is hypothesized that CNDP2 is not active in human skeletal muscle since Lenney and coworkers (1985) demonstrated that the optimum pH for CN2 is 9.5, whereas the pH of muscle does not exeed 7.4 (as described in section 3.4).

Figure 14 gives an overview of the expression of the different enzymes and transporters in the different organs. Enzymes and transporters that are shown to be expressed in human tissue are depicted in red, the ones that are until now only proven in rodents are depicted in dark red.

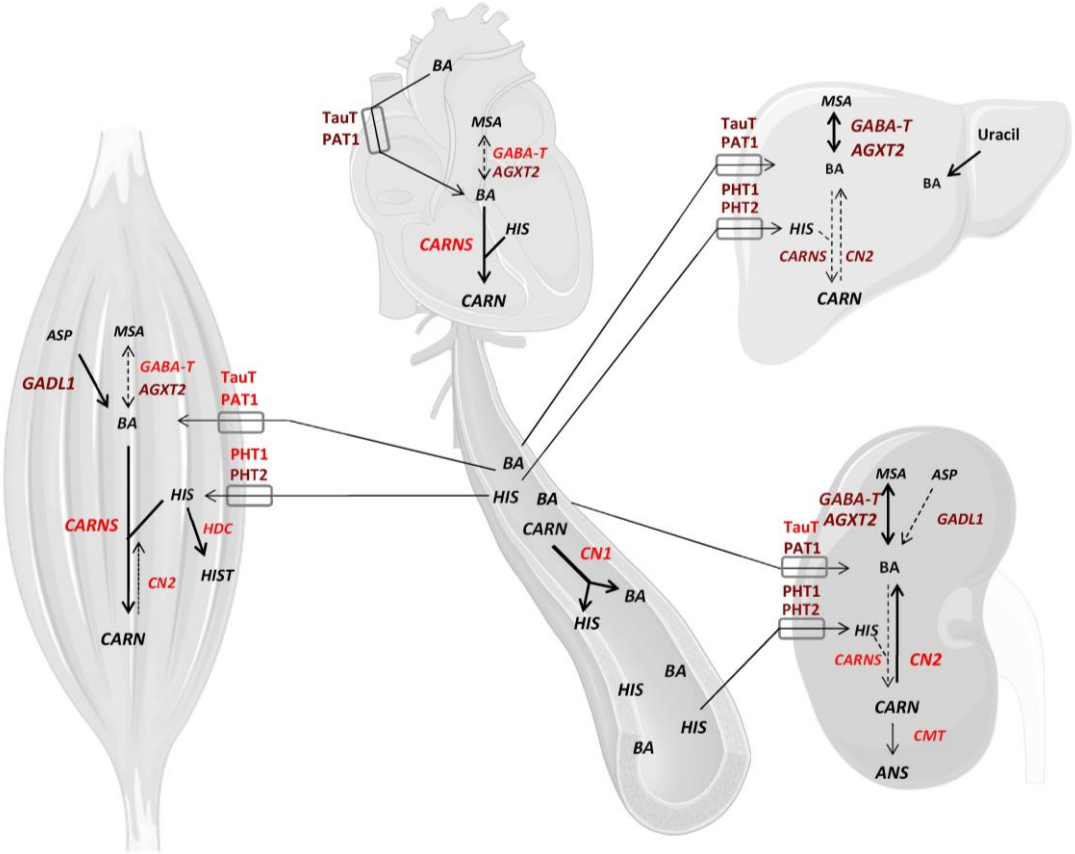

Figure 14: Schematic overview of the current understanding of the carnosine metabolism. The expression of enzymes and transporters in red is demonstrated in humans, the ones in dark red are demonstrated in rodents but either not present or not investigated in humans. Dashed lines: less important, thick lines: important reactions. ANS: anserine, ASP: aspartate, BA: beta-alanine, CARN: carnosine, HIS: L-histidine, HIST: histamine, MSA: malonate semi-aldehyde.

4.4. Tissue carnosine homeostasis versus plasma beta-alanine homeostasis?

As already mentioned, chronic oral beta-alanine supplementation has been shown to increase muscle carnosine levels, thus disturbing tissue carnosine homeostasis. In this respect, one can assume that plasma beta-alanine levels, which are enhanced by beta-alanine supplementation, are also under homeostatic control. Increased tissue carnosine levels can thus be an attempt to maintain the plasma beta-alanine set point. This rises the question **which homeostasis predominates, tissue carnosine or plasma beta-alanine**?

In section 5 and 6, we will discuss the available literature on how the above described enzymes and transporters contribute to the regulation of tissue carnosine homeostasis or plasma beta-alanine homeostasis, respectively. Because this thesis focuses on muscle metabolism, we will mainly concentrate on the effectors that are shown to be expressed inside myocytes. However, when discussing plasma beta-alanine homeostasis, other organs are assumed to also be involved in this process.

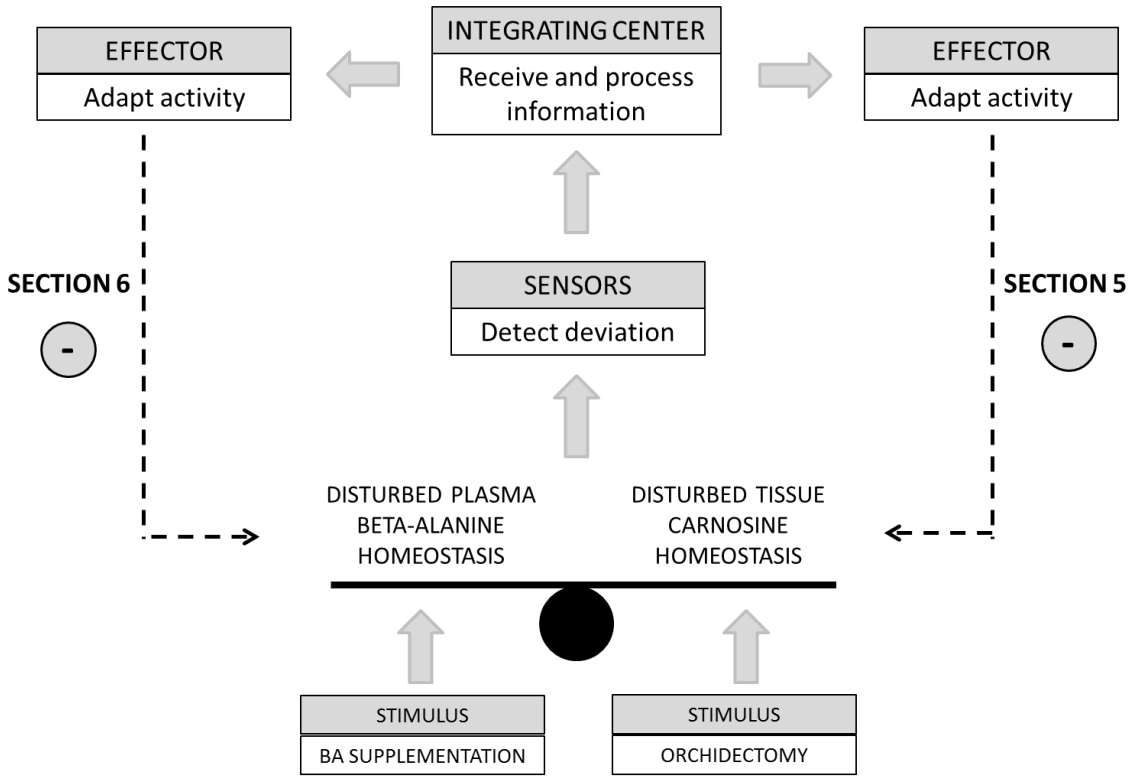

Figure 15: Graphical overview of the concept of homeostasis, applied to the regulation of tissue carnosine homeostasis and plasma beta-alanine homeostasis. Both homeostatic regulations are closely connected with each other. Disturbed homeostasis will be detected by sensors and will activate effectors to either restore carnosine or beta-alanine homeostasis, thereby antagonizing the detected disturbance. Negative feedback loops are indicated by the dashed arrow.

First, we will elaborate on how the described effectors can maintain muscle carnosine homeostasis when a fall in muscle carnosine occurs, for example after **orchidectomy**. Orchidectomy is the removal of the gonads (primary reproductive organs), e.g. castration of testicles in males, which leads to a dramatic fall in sex hormones (e.g. testosterone). If effectors are able to antagonize a fall in muscle carnosine, this supports the theory of predominance of tissue carnosine homeostasis. In section 6, available information supporting the theory of plasma beta-alanine homeostasis will be explored. In this context, we will discuss if and how the above mentioned effectors are influenced by increased plasma beta-alanine levels after **chronic beta-alanine supplementation** as an example (Fig 15).

5. Evidence supporting the existence of tissue carnosine homeostasis

In the previous section, enzymes and transporters involved in the carnosine metabolism are clarified. The following section will discuss if and how these effectors can effectively antagonize a fall in muscle carnosine, emphasizing the importance of muscle carnosine homeostasis. To antagonize a fall, increased carnosine synthesis and/or decreased carnosine degradation should be evoked. Orchidectomy in male mice is an example of a condition in which rodent muscle carnosine content is decreased (Peñafiel *et al.*, 2004). Interestingly, the study of Everaert et al. (2013*a*) investigated the mRNA expression of carnosine-related enzymes and transporters in mice tibialis anterior at 7 and 30 days after orchidectomy. Muscle carnosine and anserine levels were non-significantly decreased at 7 days following castration, and a significant decrease was found at 30 days, compared to control mice.

5.1. Increasing carnosine synthesis

In case a fall in muscle carnosine occurs, an upregulation of CARNS is necessary to compensate for the reduced intramyocellular carnosine levels and thus maintain carnosine homeostasis. Indeed, Everaert et al. (2013*a*) demonstrated that CARNS mRNA expression was significantly higher at 7 days (+43%) and 30 days (+57%) following orchidectomy. This clearly indicates that carnosine synthesis is stimulated in case a fall in muscle carnosine occurs and thus supports the importance of muscle carnosine homeostasis (Fig 15).

5.2. Decreasing carnosine and beta-alanine degradation

Next to increased carnosine synthesis, decreased carnosine and beta-alanine degradation should occur to antagonize a fall in muscle carnosine. This can be established by a downregulation of the beta-alanine degrading enzymes GABA-T and AGXT2 and tissue carnosinase (CNDP2).

5.2.1. Beta-alanine degradation

In view of maintaining muscle carnosine homeostasis, beta-alanine degradation should be downregulated since more beta-alanine is needed to increase the degree of carnosine synthesis. GABA-T mRNA expression was indeed lower 7 days following orchidectomy in

male mice (Fig 13) (Everaert *et al.*, 2013*a*), suggesting that the priority role of beta-alanine is to serve as precursor of carnosine synthesis, rather than its role as a fuel in the citric acid cycle. Whether AGXT2 mRNA expression is also influenced in this condition, was not investigated in the study of Everaert et al. (2013*a*) and thus remains to be established.

5.2.2. Carnosine degradation

If carnosine degradation would occur inside muscle cells (which is still doubtful), a decrease in the degradation rate would be expected in case of decreased carnosine levels. Indeed, Everaert et al. (2013*a*) found lower expression of CNDP2 at 7 days following orchidectomy compared to control mice, but this effect was not longer present at 30 days (Fig 16).

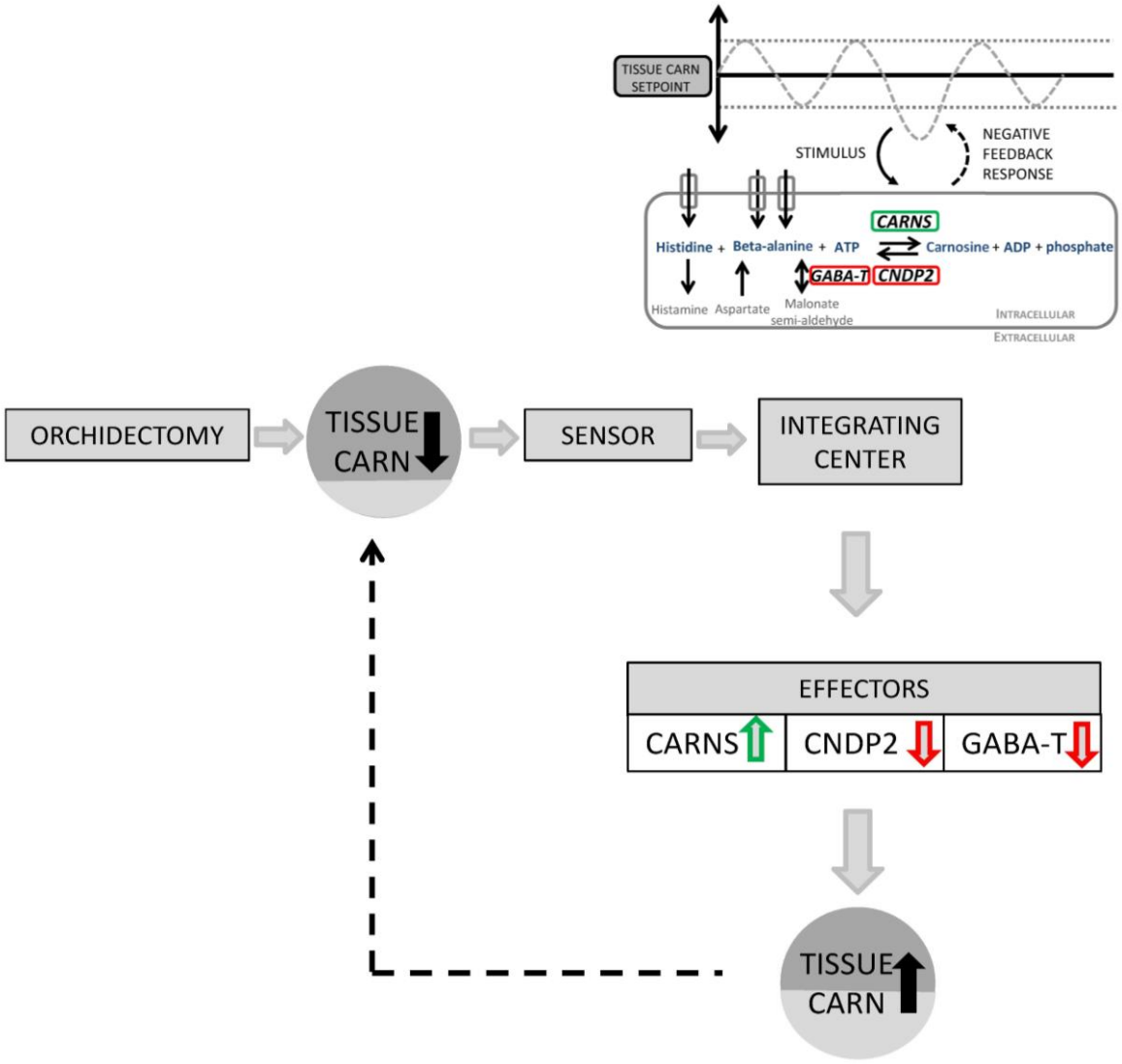

Figure 16: Summary on how tissue carnosine homeostasis is maintained following a stimulus that evokes a fall in tissue carnosine. Colors display how the expression of the enzymes and transporters is affected by the stimulus (=orchidectomy) in order to maintain tissue carnosine homeostasis (red: downregulation; green: upregulation)

In summary, the increased mRNA expression of CARNS and the decreased mRNA expression of CNDP2 and GABA-T are an indication that the muscle aims to prevent carnosine concentrations to decrease in the 7 days following orchidectomy. The altered expression of these enzymes illustrates the importance of maintaining muscle carnosine homeostasis. If the body would not consider the muscle carnosine homeostasis as an important factor, the expression of the effectors would not be affected by a fall in muscle carnosine.

6. Evidence supporting the existence of plasma beta-alanine homeostasis

The previous section demonstrated that the carnosine-related effectors expressed in myocytes can contribute to maintaining muscle carnosine homeostasis in case a fall of muscle carnosine occurs. However, since beta-alanine supplementation induces a rise in muscle carnosine, this might suggest that plasma beta-alanine homeostasis is even more important compared to muscle carnosine homeostasis. The following section will discuss if and how effectors can effectively antagonize a rise in plasma beta-alanine. If priority is given to maintaining plasma beta-alanine homeostasis, this suggests a whole body regulation rather than a pheripheral regulation on muscle level. The liver can be an important point of regulation in this process, as the hepatic portal vein carries nutrient-rich blood from the gastrointestinal tract and spleen to the liver, ensuring that ingested substances are first processed by the liver before reaching the systemic circulation. Thus, as a consequence of elevated plasma beta-alanine levels, it can be hypothesized that whole body (and especially liver) beta-alanine synthesis is decreased and beta-alanine degradation is increased.

6.1. Whole body beta-alanine synthesis

Until now, the only known endogenous pathway to synthesize beta-alanine is uracil degradation in liver. However, it is currently unclear how this pathway is regulated and whether it is affected by increased beta-alanine availability. Moreover, as described, GADL1, a specific enzyme for beta-alanine synthesis from aspartate in mammalian muscle, is only recently proposed by Liu et al. (2012*a*). GADL1 mRNA expression was found in mouse kidney and in mouse and cattle skeletal muscles, but its presence in human tissues is until now not established. Furthermore, it is currently uninvestigated whether the GADL1 expression profile is affected by increased plasma beta-alanine availability following beta-alanine supplementation. The existence of this pathway and a possible downregulation in case of increased plasma beta-alanine levels could be a demonstration that plasma beta-alanine availability controls whole body beta-alanine-related pathways.

6.2. Whole body beta-alanine degradation

Both beta-alanine transaminating enzymes GABA-T and AGXT2 are known to be highly expressed in kidney and liver, and GABA-T mRNA expression was also found, although at

a lower level, in mice skeletal muscle (Everaert *et al.*, 2013*a*), suggesting that beta-alanine might also be peripherally degraded (inside muscle cells), next to more central degradation in liver and kidney. Until now, the expression of AGXT2 in liver and kidney following beta-alanine supplementation is not yet investigated, but beta-alanine supplementation in rats resulted in increased mRNA expression of GABA-T in kidney (Ito *et al.*, 2001). Similarly, muscle GABA-T mRNA expression was shown to be upregulated (+40%) in mice supplemented with beta-alanine supplementation (Everaert *et al.*, 2013*a*). These observations demonstrate that increased plasma beta-alanine availability is antagonized by a higher degree of beta-alanine degradation in different tissues of the body (Fig 17).

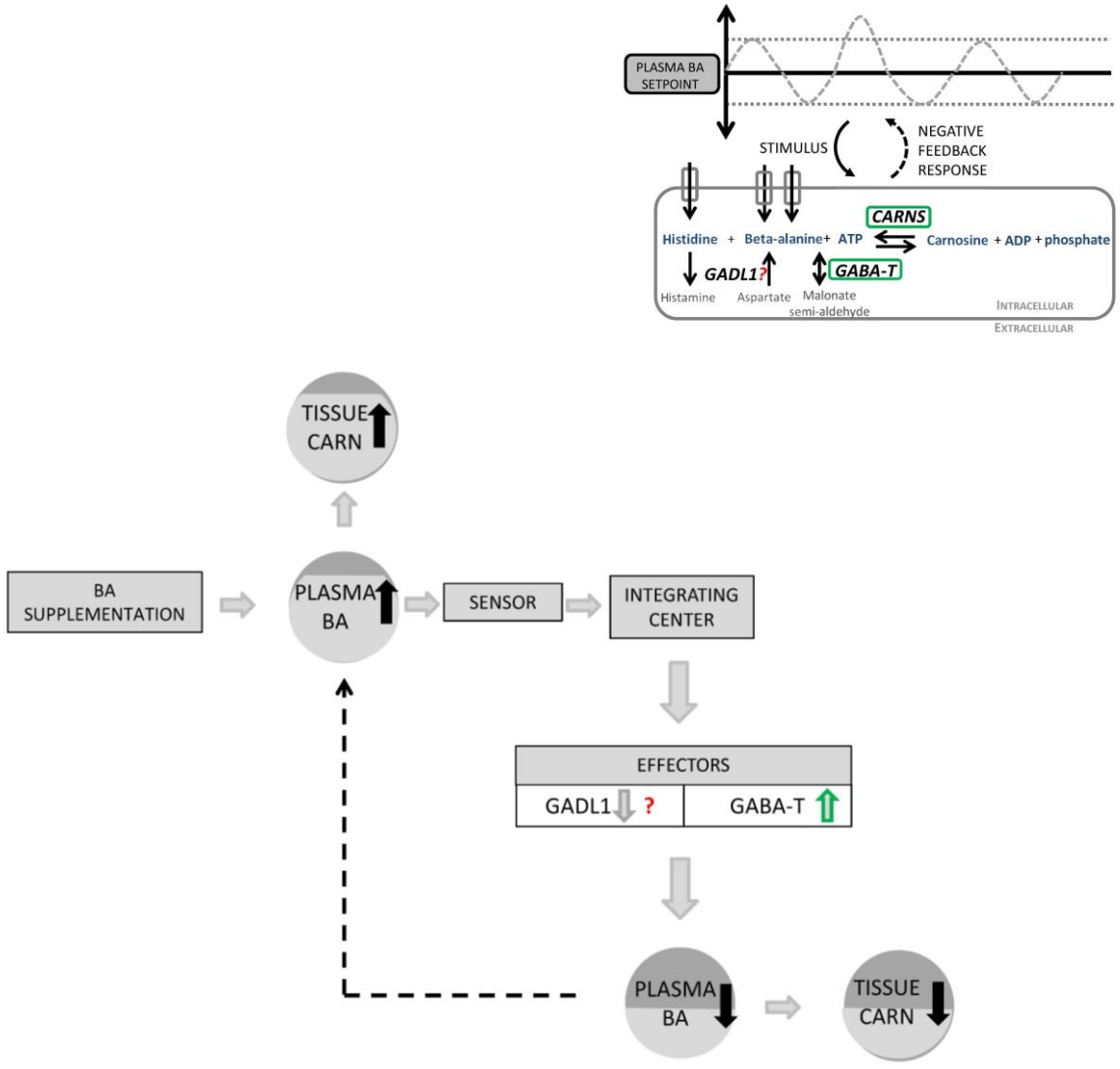

Figure 17: Summary on how plasma beta-alanine homeostasis is maintained following a stimulus that evokes a rise in plasma beta-alanine levels. Colors display how the expression of the enzymes and transporters is affected by the stimulus (=beta-alanine supplementation) in order to maintain plasma beta-alanine homeostasis (red: downregulation; green: upregulation)

In summary, the high expression of beta-alanine degrading enzymes GABA-T and AGXT2 in the central organs (liver and kidney) suggest that these enzymes are responsible for the regulation of plasma beta-alanine homeostasis. Moreover, the upregulation of GABA-T mRNA levels after beta-alanine supplementation in both kidney and muscle illustrates that increased plasma beta-alanine levels are antagonized by this pathway.

6.3. Predominance of plasma beta-alanine vs tissue carnosine homeostasis?

Based on the observations described in the previous sections, we can currently define two working hypothesis which can shortly be defined as 'tissue carnosine homeostasis' vs 'plasma beta-alanine homeostasis'. Although both homeostatic systems probably exist, no direct evidence currently exists on which of these homeostatic systems is the most important one. However, some findings may suggest that plasma beta-alanine homeostasis is the predominant one. As an example, TauT and PAT1 are shown to be expressed in skeletal muscle of both rodents and humans. Interestingly, TauT mRNA levels were higher in beta-alanine and carnosine supplemented mice (+28% and +21%, respectively) (Everaert *et al.*, 2013*a*). Because increased circulating beta-alanine concentrations stimulate the gene expression of TauT, this signifies that the large muscle organ helps to eliminate beta-alanine from the blood, which is in line with maintaining plasma beta-alanine homeostasis. If muscle carnosine homeostasis would be the main concern, transsarcolemmal beta-alanine uptake would be decreased to antagonize a rise in muscle carnosine upon beta-alanine supplementation. Moreover, in accordance with TauT, CARNS mRNA content was significantly higher in chronically beta-alanine supplemented mice and after the ingestion of one acute dose of beta-alanine (Miyaji *et al.*, 2012; Everaert *et al.*, 2013*a*). Thus, both transsarcolemmal beta-alanine uptake and intramyocellular carnosine synthesis seem to be stimulated by increased plasma beta-alanine levels, suggesting that plasma beta-alanine homeostasis predominates over tissue carnosine homeostasis. Up until now, it remains to be investigated whether TauT and CARNS are also upregulated in human skeletal muscle following beta-alanine supplementation.

Aims: the mRNA expression of all carnosine-related enzymes and transporters is already investigated in both mice and human skeletal muscle, but the effect of beta-alanine supplementation on the expression profiles are until now only explored in rodents. In

order to get a better understanding of the human plasma beta-alanine and tissue carnosine homeostatic regulation, this thesis aims to unravel the expression profiles of carnosine-related enzymes and transporters following chronic ingestion of beta-alanine.

7. Homeostatic failure by beta-alanine supplementation

Although the above described information suggests that tissue carnosine homeostasis and plasma beta-alanine homeostasis is kept as stable as possible by a complex interplay of the different effectors, there is a specific situation in which both homeostatic processes are disturbed, i.e beta-alanine supplementation. As mentioned, chronic beta-alanine supplementation was shown to increase intramuscular carnosine concentrations above the normal range. Shortly thereafter, carnosine loading was demonstrated to augment fatigue threshold and improve high-intensity exercise performance (Hill *et al.*, 2007; Hobson *et al.*, 2012; Bellinger, 2014). These findings led to a fast-growing interest for beta-alanine as a nutritional supplement for competitive athletes participating in a range of sports. A recent meta-analysis bundled all studies investigating beta-alanine supplementation and exercise performance, providing evidence that exercise of 0.5-10min in duration is the time frame in which beta-alanine supplementation is the most beneficial (Saunders *et al.*, 2016). The currently used beta-alanine supplementation protocol will be discussed together with the available information on determinants of this loading process. Interestingly, supplemented beta-alanine was shown to have a low incorporation efficiency into muscle carnosine, demonstrating that the body is equipped with other pathways for beta-alanine and thus to a certain extent tries to keep muscle carnosine between homeostatic limits. Increased muscle carnosine concentrations by chronic beta-alanine supplementation can thus be seen as failure of the body to maintain plasma beta-alanine homeostasis, and thus subsequently also a failure to keep muscle carnosine within the normal range.

7.1.1. Beta-alanine supplementation protocol

Based on different supplementation studies, it was already shown that 4 - 6.4g beta-alanine per day during 4-10 weeks increases carnosine concentrations by 40-80% in both trained and untrained individuals (Baguet *et al.*, 2009; Stellingwerff *et al.*, 2012*a*). Stellingwerf et al. (2012*a*) identified a linear dose–response relationship between beta-alanine intake and carnosine loading, suggesting that the total amount of supplemented beta-alanine is an important determinant of the degree of muscle carnosine loading. This finding would appear to make the prescriptive application of beta-alanine quite simple. However, the daily dose of beta-alanine was limited in the first supplementation studies

because doses larger than 800mg (10mg/kg body weight) were accompanied by moderate to severe parasthesia symptoms (prickly sensation on the skin) (Harris *et al.*, 2006). To circumvent these undesirable symptoms, multiple beta-alanine doses (maximal 800mg) are supplemented throughout the day to achieve the desired total beta-alanine dose daily. More recently, a slow-release beta-alanine tablet form has become commercially available as another strategy to circumvent parasthesia symptoms. The slow-release beta-alanine tablet was shown to result in slower absorption kinetics, making it possible to ingest 1.6g of slow-release beta-alanine without perceiving side-effects (Décombaz *et al.*, 2012). This allows for larger daily doses (up to 6.4g) to be ingested throughout a supplementation period. Daily doses larger than 6.4 g/day are yet to be examined, as well as prolonged beta-alanine supplemention (more than 3 months).

7.1.2. Metabolic fate on ingested beta-alanine

Considering the high baseline muscle carnosine concentrations, an increase of 40-80% is a significant amount of loading (e.g., 24-35mmol/kg dry weight in vastus lateralis muscle after 4 weeks of supplementation (Harris *et al.*, 2006)). However, the current supplementation protocol requires athletes to take a large dose of beta-alanine each day during several weeks. In sport settings, it would be more useful if athletes could load their carnosine levels in a shorter time in anticipation of a championship or competition. To optimize the beta-alanine loading protocol, the **efficiency of beta-alanine supplementation** can be calculated by dividing the molar increase in muscle carnosine by the total ingested molar amount of beta-alanine. Surprisingly, Stegen et al (2013*a*) found that this efficiency is very low (around 2.8% when assuming that 40% of body mass is muscle mass), meaning that only 2-3% of the total ingested amount of beta-alanine is actually incorporated into muscle carnosine. It was shown that a small part of chronically ingested beta-alanine in excreted in the urine (1-2%), indicating that approximately 95% of ingested beta-alanine is not used for carnosine synthesis and thus has another metabolic fate in the human body (Stegen *et al.*, 2013*a*). Uptake of beta-alanine into non-muscle tissue (such as nervous tissue) may account for a (small) portion of the available beta-alanine after ingestion. Table 3 gives an overview of the efficiency of beta-alanine or carnosine supplementation in different published studies in literature.

Table 3: Overview of studies investigating the effect of beta-alanine or carnosine supplementation on human muscle carnosine loading and the efficiency of the different supplementation protocols. Efficiency is calculated by dividing the total molar increase in muscle carnosine (40% of body weight x Δ[muscle carnosine]) by the total ingested molar amount of beta-alanine or carnosine. BA: beta-alanine; CARN: carnosine; GASTR: gastrocnemius; SOL: soleus; VI: vastus intermedius; VL: vastus lateralis

Study	Daily BA dose (g)	Length	Total BA dose (g)	Muscle	CARN increase (mmol/kg ww)	CARN increase (%)	Efficiency (%)
Harris (2006)	3.2	4 wks	89.6	VL	1.95	39.8	6.17
	4 - 6.4	4 wks	145.6	VL	2.76	45.6	5.37
Hill (2007)	4 – 6.4	4 wks	145.6	SOL	2.55	51.3	5.0
	4 – 6.4	10 wks	414.4	GASTR	3.7	74.4	2.55
Derave (2007)	2.4 – 4.8	4-5 wks	139.2	SOL	3.63	46.7	7.02
				GASTR	3.74	37.0	
Kendrick (2008)	6.4	4 wks	179.2	VL	3.2	53.5	3.85
Kendrick (2009)	6.4	4 wks	179.2	VL	2.04	34.8	2.45
Baguet (2009)	2.4 – 4.8	5-6 wks	180	SOL	2.2	39.0	2.68
				GASTR	1.79	23.4	
				TA	1.68	26.9	
Baguet (2010*a*)	5	7 wks	245	SOL	1.42	45.3	1.65
				GASTR	1.29	28.2	
Stellingwerff (2012*a*)	1.6 – 3.2	8 wks	134.4	GASTR	2.36	44.5	5.45
				TA	2.45	30.3	
Stegen (2013)	3.2	46 days	147.2	SOL	1.7	48.6	2.71
				GASTR	1.67	36.7	
Bex (2013)	6.4	23 days	147.2	SOL	2.64	57.8	4.85
				GASTR	2.78	40.2	
Bex (2014)	6.4	23 days	147.2	SOL	2.95	57.8	5.92
				GASTR	3.27	37.7	
Danaher (2014)	4.8 – 6.4	6 wks	224	SOL	4.92	88	6.32
				GASTR	5.03	62	
Chung (2014)	6.4	6 wks	268.8	SOL	5.2	161	5.65
				GASTR	5.7	143	
Gross (2014)	3.2	38 days	121.6	GASTR	2	22.7	5.02
				TA	2.5	36.2	
				VI	2.6	46.4	
				VL	1.8	24.3	
Cochran (2015)	3.2	4 wks	89.6	VL	2.1	32.8	7.2
	3.2	10 wks	224	VL	3.3	51.6	4.5
Study	**Daily CARN dose (g)**	**Length**	**Total CARN dose (g)**	**Muscle**	**CARN increase (mmol/kg ww)**	**CARN increase (%)**	**Efficiency (%)**
Harris (2006)	10 - 16	4 wks	364	VL	4.1	70.7	8.1

In line with this, Pihl & Fritzson (1955) already reported that more than 90% of the injected C^{14}-labelled beta-alanine in rats was recovered in the expired CO_2 in 5h, suggesting that beta-alanine can be metabolized elsewhere, most probably as a carbon source for energy provision through oxidation. As described above, it could be hypothesized that beta-alanine is oxidized by GABA-T and/or AGXT2 to form malonate semi-aldehyde which can ultimately enter the citric acid cycle and thus contribute to total energy delivery. Thus, one could assume that the beta-alanine degrading pathway leads to loss of beta-alanine, which could be a possible explanation of the low efficiency of beta-alanine supplementation. However, up until now, no studies are available investigating the contribution of these enzymes to the circulating beta-alanine and muscle carnosine metabolism. Based on this information, it could be questioned whether blocking GABA-T and AGXT2 could be a way to decrease the amount of beta-alanine degradation, thereby stimulating higher plasma beta-alanine levels and thus subsequently promote muscle carnosine synthesis (higher tissue carnosine synthesis as a consequence of a more disturbed plasma beta-alanine homeostasis).

Aims: GABA-T and AGXT2 are two enzymes suggested to transaminate beta-alanine. This thesis aims to investigate whether these enzymes are indeed involved in plasma beta-alanine and tissue carnosine homeostasis. Therefore, combined oral beta-alanine supplementation with inhibitors of GABA-T and/or AGXT2 activity is hypothesized to cause a more disturbed plasma beta-alanine homeostasis and thus lead to a higher amount of tissue carnosine laoding.

7.1.3. Determinants of carnosine loading

Few studies recently revealed some determinants of muscle carnosine loading in order to increase the efficiency of the beta-alanine supplementation protocol. First, Stegen et al. (2013*a*) showed that **coingesting beta-alanine with a meal** containing carbohydrates and proteins is able to enhance the amount of carnosine loading compared to ingesting beta-alanine between the meals. This observation suggests that insulin could play a stimulating role in one or more crucial steps of the muscle carnosine synthesis process. In addition, **training and/or training status** is another determinant of muscle carnosine loading. Bex et al. (2014) demonstrated that trained muscles had approximately two-fold higher carnosine loading compared with untrained muscles for the same oral beta-alanine

supplementation protocol. This observation was found both within athletes and between athletes participating in different sports. Furthemore, in a follow-up study, it was shown that carnosine loading effectiveness was improved by implementing high-volume and high-intensity training protocols in combination with beta-alanine in non-specifically trained subjects (Bex *et al.*, 2015).

Table 4: Summary of the efficiency of beta-alanine supplementation for different supplementation studies, showing that meal co-ingestion, exercise training (both chronic and acute) and high daily intakes during a limited period lead to highest efficiency of beta-alanine induced carnosine loading. Adapted from Blancquaert et al. (2015). BA: beta-alanine

Study	Dose	Meal	Training	BA form	Efficiency (%)
Stegen et al. (2013)	3.2g/d, 46d	-	-	pure	2.39
Stegen et al. (2013)	3.2g/d, 46d	+	-	slow-release	2.76
Stegen et al. (2013)	3.2g/d, 46d	+	-	pure	2.99
Bex et al. (2014)	6.4g/d, 23d	+	-	slow-release	3.49
Bex et al. (2015)	6.4g/d, 23d	+	+ acute	slow-release	5.42
Chung et al. (2014)	6.4g/d, 42d	+	+ chronic	slow-release	5.65
Bex et al. (2014)	6.4g/d, 23d	+	+ chronic	slow-release	5.82

During prolonged competition, it is of importance for athletes to effectively **maintain muscle carnosine concentrations** at elevated levels. Stegen et al. (2014) demonstrated that a dose of 1.2g/day is optimal to keep muscle carnosine content elevated at 30-50% above baseline after a loading phase.

Despite the strategies that are shown to improve the efficiency of chronic beta-alanine supplementation (Table 4), this **efficiency of beta-alanine supplementation remains low** with ~90% of the beta-alanine still having an unknown metabolic fate. Moreover, the study of Hill et al. (2007) demonstrated that loading efficiency is decreasing throughout the supplementation period. In their study, carnosine loading was measured in the vastus lateralis after 4 and 10 weeks of beta-alanine supplementation, which corresponds to a total ingested beta-alanine dose of 145.6g and 414.4g, respectively. They reported an increase in carnosine levels of 58.8% at 4 weeks and 80.1% at 10 weeks, demonstrating a slower loading process and thus lower loading efficiency in the second period of

supplementation (incorporation efficiency for beta-alanine was 5.0% in first 4 weeks vs 1.22% in subsequent 6 weeks). This reduced efficiency over time suggests that, during the course of supplementation, the conditions for carnosine synthesis are less optimal. This could be caused by an attempt of the body to not further disturb muscle carnosine homeostasis above a certain point (reaching a plateau), but it could also be hypothesized that beta-alanine does not longer remain the (only) driving factor for carnosine synthesis. As mentioned, L-histidine is the accompanying amino acids for carnosine synthesis. Until now, no study investigated the effect of long term beta-alanine supplementation on body and muscle L-histidine concentrations. The reduced efficiency of beta-alanine supplementation may therefore result from the scarcity of L-histidine following a long and intensive beta-alanine supplementation protocol.

Aims: Beta-alanine is widely accepted as the rate-limiting factor in the carnosine synthesis process. Therefore, controlling beta-alanine availability is considered as the main factor to regulate carnosine synthesis. However, the beta-alanine supplementation protocol that is currently used is long and very intensive while no attention is assigned to L-histidine availability. This PhD thesis aims to explore the effect of chronic oral beta-alanine supplementation on L-histidine availability, thereby revealing the possible contributing role of L-histidine to the carnosine synthesis process.

8. Experimental aims and outline of the thesis

Carnosine is the main molecule investigated in this thesis. It has several biochemical and physiological properties relevant to both exercise performance and health-related issues. Although carnosine is extensively investigated during the past decade, its specific metabolism is still far from fully understood. Figure 18 gives an overview of the studies included in this thesis. These studies were performed to contribute to a better understanding of the unresolved physiological puzzle named 'regulation of muscle carnosine homeostasis'.

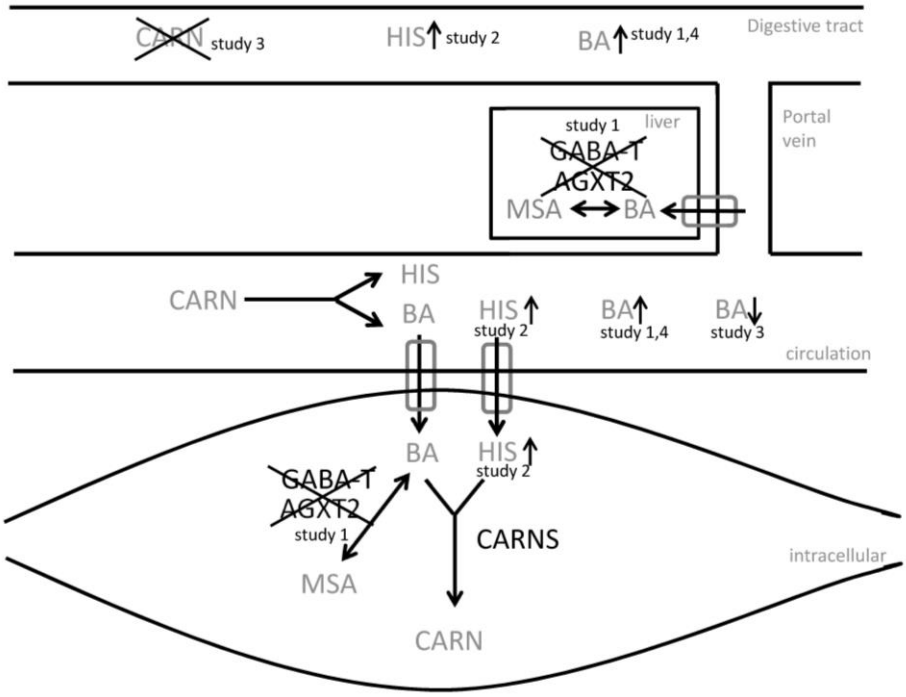

Figure 18: Schematic overview of the studies included in this thesis

Carnosine loading by beta-alanine supplementation is a rather inefficient process, which is probably a consequence of the strong homeostatic system, as described above. Study 1 and 2 focus on strategies to improve this supplementation efficiency by gathering a better insight in the carnosine metabolism. **Study 1** mainly focused on the role of beta-alanine transaminases in the regulation of muscle carnosine levels upon beta-alanine supplementation. As it is hypothesized that ingested beta-alanine can be metabolized elsewhere, a possible pathway is transamination by GABA-T and AGXT2 in either liver and/or kidney or inside myocytes. It is therefore investigated whether inhibiting the beta-

alanine transaminase pathway is an efficient way to enhance the amount of carnosine loading. In **study 2**, it was questioned whether beta-alanine is indeed the one and only rate-limiting factor for carnosine synthesis. In some animals, L-histidine was shown to be rate-limiting over beta-alanine (Tamaki *et al.*, 1977; Park *et al.*, 2013). Whether carnosine loading efficiency can thus be enhanced by L-histidine supplementation (alone or combined with beta-alanine) is explored in study 2. To gather a better understanding of the carnosine metabolism, study 3 and 4 examined two extreme situations, namely a lack of any dietary beta-alanine intake on the one hand and chronic beta-alanine supplementation on the other hand, in order to clarify the effect of these scenarios on the carnosine homeostasis and its regulation. The effect of a 6-month vegetarian diet in previous omnivorous subjects on the homeostasis of three carninutrients (carnosine, carnitine, creatine) was examined in **study 3**. Lastly, **study 4** investigated the transcriptional events of carnosine-related enzymes and transporters in human skeletal muscles in response to beta-alanine supplementation to further elucidate how muscle carnosine homeostasis is disturbed and maintained.

The aims and underlying hypotheses investigated in this thesis are summarized below:

- To elucidate if beta-alanine transaminases are involved in the regulation of muscle carnosine concentrations upon beta-alanine supplementation

  - It is hypothesized that beta-alanine is degraded to malonate semi-aldehyde by GABA-T and/or AGXT2 (study 1). If so, inhibiting this transaminase pathway could be a way to evoke a higher amount of carnosine loading following beta-alanine supplementation (study 1)

  - Beta-alanine transaminases are mainly known to be expressed in central organs (liver and kidney), but they might also be involved in beta-alanine degradation inside myocytes (study 1). It is therefore hypothesized that they are upregulated by beta-alanine supplementation (study 4)

- To explore whether beta-alanine is the only factor driving carnosine homeostasis

  - Beta-alanine is considered as the rate-limiting precursor for carnosine synthesis in humans. The effect of L-histidine supplementation on human muscle carnosine

loading is until now never explored. In some animal species, however, L-histidine is shown to be the rate-limiting factor in the carnosine synthesis process. It can be expected that L-histidine supplementation in humans can thus also influence muscle carnosine concentrations (study 2)

- Based on the low efficiency of beta-alanine supplementation, we hypothesize that intramyocellular L-histidine levels are depleted by beta-alanine supplementation, thus co-supplementation of L-histidine with beta-alanine can improve the loading efficiency (study 2)

- To gain more insight in the determinants, manipulation and regulation of muscle carnosine homeostasis

- Cross-sectional data demonstrated that vegetarians have somewhat lower muscle carnosine levels compared to omnivores, suggesting that the diet (containing HCDs and thus beta-alanine) is a determinant of baseline muscle carnosine homeostasis. The same cross-sectional findings are reported for plasma and muscle carnitine and creatine concentrations. By switching omnivores onto a vegetarian diet for 6 months, we hypothesize that homeostasis of carnosine (and other carninutrients carnitine and creatine) is disturbed, confirming that the diet is a determinant of baseline muscle carninutrients homeostasis (study 3)

- We know that plasma beta-alanine homeostasis is disturbed by chronic beta-alanine supplementation. We hypothesize that, as a consequence of disturbed plasma beta-alanine levels, muscle carnosine levels are increased to remove the redundant plasma beta-alanine (study 1). We thus hypothesize that fasted plasma beta-alanine levels upon beta-alanine supplementation can be a suitable predictor for the amount of carnosine loading (study 1, 2, 3)

- We hypothesize that some known and putative players in the metabolic pathways of carnosine are expressed in human skeletal muscle such as beta-alanine synthezing enzyme GADL1, beta-alanine degrading enzyme GABA-T and histidine decarboxylase (HDC) (study 4). It was furthermore expected that, alike rodents, human muscle carnosine disturbance is mainly accomplished by changes in the

gene expression of both the beta-alanine transporters and carnosine synthase enzyme (study 4)

II

Original Research

Study 1

Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta-alanine transamination

Blancquaert L, Baba SP, Kwiatkowski S, Stautemas J, Stegen S, Barbaresi S, Chung W, Boakye AA, Hoetker JD, Bhatnagar A, Delanghe J, Vanheel B, Veiga-da-Cunha M, Derave W, Everaert I

J Physiol (2016) 594(17): 4849-63

KEY POINTS

- Using recombinant DNA technology, the present study provides the first strong and direct evidence indicating that beta-alanine is an efficient substrate for the mammalian transaminating enzymes 4-aminobutyrate-2-oxoglutarate transaminase and alanine-glyoxylate transaminase.
- The concentration of carnosine and anserine in murine skeletal and heart muscle depends on circulating availability of beta-alanine, which is in turn controlled by degradation of beta-alanine in liver and kidney.
- Chronic oral beta-alanine supplementation is a popular ergogenic strategy in sports because it can increase the intracellular carnosine concentration and subsequently improve the performance of high-intensity exercises. The present study can partly explain why the beta-alanine supplementation protocol is so inefficient, by demonstrating that exogenous beta-alanine can be effectively routed toward oxidation.

ABSTRACT

The metabolic fate of orally ingested beta-alanine is largely unknown. Chronic beta-alanine supplementation is becoming increasingly popular for improving high-intensity exercise performance because it is the rate-limiting precursor of the dipeptide carnosine (beta-alanyl-L-histidine) in muscle. However, only a small fraction (3–6%) of the ingested beta-alanine is used for carnosine synthesis. Thus, the present study aimed to investigate the putative contribution of two beta-alanine transamination enzymes, namely 4-aminobutyrate-2-oxoglutarate transaminase (GABA-T) and alanine-glyoxylate transaminase (AGXT2), to the homeostasis of carnosine and its methylated analogue anserine.We found that, when transfected into HEK293T cells, recombinant mouse and human GABA-T and AGXT2 are able to transaminate beta-alanine efficiently. The reaction catalysed by GABA-T is inhibited by vigabatrin, whereas both GABA-T and AGXT2 activity is inhibited by aminooxyacetic acid (AOA). Both GABA-T and AGXT2 are highly expressed in the mouse liver and kidney and the administration of the inhibitors effectively reduced their enzyme activity in liver (GABA-T for vigabatrin; GABA-T and AGXT2 for AOA). In vivo, injection of AOA in C57BL/6 mice placed on beta-alanine (0.1% w/v in drinking water) for 2 weeks lead to a 3-fold increase in circulating beta-alanine levels and to significantly

higher levels of carnosine and anserine in skeletal muscle and heart. By contrast, specific inhibition of GABA-T by vigabatrin did not affect carnosine and anserine levels in either tissue. Collectively, these data demonstrate that homeostasis of carnosine and anserine in mammalian skeletal muscle and heart is controlled by circulating beta-alanine levels, which are suppressed by hepatic and renal beta-alanine transamination upon oral beta-alanine intake.

ABBREVIATIONS LIST

AGXT2, alanine-glyoxylate transaminase; AOA, aminooxyacetate; CARNS, carnosine synthase; GABA-T, 4-aminobutyrate-2-oxoglutarate transaminase; HCD, histidine-containing dipeptide; MSA, malonate semi-aldehyde; PBS, phosphate-buffered saline; SAL, saline; TauT, taurine transporter

INTRODUCTION

Carnosine is a versatile dipeptide, composed of beta-alanine and L-histidine. Anserine (beta-alanyl-Nπ-methylhistidine) and ophidine/balenine (beta-alanyl-Nτ-methylhistidine) are two methylated analogues of carnosine, collectively called histidine-containing dipeptides (HCDs). HCDs are mainly present in mammalian skeletal muscle and neuronal tissue and, to a smaller extent, in the heart, liver and kidney (Boldyrev *et al.*, 2013). Skeletal muscles of all mammals, except humans, possess both carnosine and a methylated analogue (anserine or ophidine). In human muscles, carnosine is the only HCD (5-8mM), with ~2-fold higher concentrations in fast-twitch fibers than slow-twitch fibers (Harris *et al.*, 1998; Kendrick *et al.*, 2009). Several physiological properties of carnosine are relevant to muscular function and homeostasis, such as pH buffering, anti-oxidant capacity, increasing Ca^{2+} sensitivity and inhibiting protein glycation (Boldyrev *et al.*, 2013; Blancquaert *et al.*, 2015).

The major pathways involved in carnosine metabolism are synthesis from and hydrolysis to its constituent amino acids, by carnosine synthase (CARNS) (Drozak *et al.*, 2010) and carnosinases (CN) (Teufel *et al.*, 2003), respectively. Beta-alanine has been shown to be the rate-limiting precursor for carnosine synthesis in human muscle cells (Harris *et al.*, 2006). Because HCDs are present in meat and fish, the daily dietary intake of these dipeptides in an omnivorous diet is considered to affect the availability of beta-alanine and therefore possibly also the muscle carnosine content. Accordingly, chronic oral beta-alanine supplementation (4-6g/day during 4-10 weeks) was found to increase muscle carnosine content by 40-80% (Harris *et al.*, 2006; Hill *et al.*, 2007; Baguet *et al.*, 2009). By contrast, a vegetarian diet is free of HCDs and long-term vegetarians may have somewhat lower muscle carnosine contents compared to omnivores (Everaert *et al.*, 2011).

However, considering the high amounts of HCDs present in mammalian muscles and the significant roles that they fulfill, carnosine and anserine homeostasis probably do not depend entirely on the nutritional supply of beta-alanine. Moreover, herbivores also show a high muscle HCD content (Dunnett & Harris, 1999; Boldyrev *et al.*, 2013), although both beta-alanine and carnosine are absent in plants and, consequently, from the herbivorous diet. This implies the existence of endogenous pathways that synthesize

beta-alanine, such as uracil degradation. Similarly, pathways may exist to degrade beta-alanine and maintain beta-alanine levels and, subsequently, HCD levels within homeostatic limits.

Under conditions where exogenous beta-alanine supply exceeds the need and/or capacity to synthesize HCDs, beta-alanine is probably degraded and used as an energy source. This was recently suggested by Stegen *et al* (2013), who found that daily orally ingested beta-alanine as an ergogenic supplement has a very high whole body retention (only <2% was excreted in urine) and only a small fraction of the exogenous beta-alanine is taken up by the human muscles to be converted into carnosine (3-6%). Moreover, Pihl & Fritzson (1955) reported that more than 90% of the injected C^{14}-labelled beta-alanine in rats was recovered in the expired CO_2 in 5 h, suggesting that beta-alanine can be metabolized elsewhere, most probably as a carbon source for energy provision through oxidation. As a result of this, beta-alanine supplementation, which recently became very popular among athletic populations as a result of its ergogenic potential (Hill *et al.*, 2007; Derave *et al.*, 2007), is a rather impractical process, requiring athletes to take large doses of beta-alanine every day over several weeks to induce HCD loading, resulting in a total ingested dose that is an order of magnitude higher than the genuine amount of beta-alanine required to synthesize dipeptides.

To enter the citric acid cycle and provide energy, the amine group of beta-alanine can be removed through a transamination resulting in the formation of the keto-acid malonate semi-aldehyde (MSA). Mostly based on enzymatic assays in cell extracts, two mitochondrial enzymes are known to catalyse this reaction: 4-aminobutyrate-2-oxoglutarate transaminase (EC 2.6.1.19; also known as GABA-T or beta-alanine-2-oxoglutarate transaminase) (Ito *et al.*, 2001) and alanine-glyoxylate transaminase (EC 2.6.1.44; also known as AGXT2 or beta-alanine-pyruvate transaminase) (Rodionov *et al.*, 2014). Vigabatrin is a known selective irreversible inhibitor of GABA-T (Lippert *et al.*, 1977), whereas aminooxyacetate (AOA) is known to inhibit all pyridoxal-5'-phosphate-dependent enzymes, including GABA-T and AGXT2 (John *et al.*, 1978; Tamaki *et al.*, 1990; Horváth & Wanders, 1995). Interestingly, administration of AOA has already been reported to increase urinary, liver, kidney and plasma beta-alanine levels in rats by 27-, 15-, 10- and 3-fold, respectively (Baxter & Roberts, 1961; Kurozumi *et al.*, 1999). These

results suggest that inhibiting both the transaminase enzymes (GABA-T and AGXT2) might be an efficient strategy for counteracting the beta-alanine catabolism in rodents. However, to date, the effect of AOA on tissue HCD levels and the effect of vigabatrin on both beta-alanine and HCD metabolism have not been determined.

The present study aimed to test the hypothesis that beta-alanine is degraded by the transaminase enzymes GABA-T and AGXT2 and that this reaction regulates tissue HCD homeostasis. The present study first aimed to demonstrate that beta-alanine is a suitable substrate for both transaminase enzymes by means of recombinant DNA technology, and also that vigabatrin is an inhibitor of GABA-T, whereas AOA inhibits both GABA-T and AGXT2 activity towards beta-alanine. In addition, the study aimed to determine the tissue mRNA expression of beta-alanine transaminases and their role in muscle HCD metabolism upon oral beta-alanine intake.

MATERIALS AND METHODS

Ethical approval

The experimental protocol was approved by the Ethics Committee for Animal Research at Ghent University and followed the Principles of Laboratory Animal Care.

Part 1: in vitro enzymatic experiments

Cloning and expression of mouse GABA-T and AGXT2 in HEK293T cells

GABA-T and AGXT2 were PCR-amplified using cDNA from mouse liver using Phusion High-Fidelity DNA Polymerase, cloned in pEF6/myc-HisA plasmid and expressed in HEK293T cells as C-terminal His_6-tagged proteins as described previously (Veiga-da-Cunha et al., 2014). HEK293T cell extracts from three independent experiments (n=3) were prepared 48 h after transfection, by removing the medium, washing the plates with phosphate-buffered saline (PBS) and collecting the cells from each plate in 0.5 ml of extraction buffer without Triton X-100. The cells were then lysed by freezing twice in liquid nitrogen and genomic DNA was removed by treating the lysates with DNase I (125 U/ml). The extracts were stored at -80°C before analysis of the recombinant proteins by SDS-PAGE/Western-blotting and measurement of enzymatic activities.

Assays of GABA-T and AGXT2 transaminase activities

GABA-T activity was measured using a spectrophotometric assay based on the sequential transamination and glutamate dehydrogenase reaction, which couples the reduction of iodonitrotetrazolium to a purple iodonitrotetrazolium-formazan dye that absorbs at 490 nm. The reaction was followed at 30°C in a mixture (1 ml) containing 50 mM Tris (pH 8.5), 1 mM ADP-Mg2+, 5 µM pyridoxal-phosphate, 5 mM MgCl2, 2 mM EGTA, 1 mM NAD+, 0.5 mg/ml bovine serum albumin (BSA), 75 µM INT, 1 mM α-ketoglutarate, 2.5 mM ɣ-aminobutyric acid (GABA) or beta-alanine, 1.5 U of recombinant diaphorase from *Clostridium kluyveri* (500 U/ml) and 10 U of beef liver glutamate dehydrogenase (5000 U/ml). Vigabatrin (0.5 mM) and AOA (2 µM) were added to the activity assay and the reaction was started by the addition of HEK293T cell extracts. Appropriate blanks in the absence of GABA or beta-alanine were run in parallel. The concentrated stock of diaphorase that was used in the assay (10 mg/ml) was prepared in 50% glycerol, 0.2 M Tris (pH 7), 0.54 mM flavin mononucleotide and 0.25 mg/ml BSA and stored at -20°C.

AGXT2 activity was measured in a two-step assay using alanine dehydrogenase to measure L-alanine formed during the AGXT2 transamination of DL-β-aminoisobutyrate (or beta-alanine) in the presence of pyruvate. In the first step (0.2 ml) the assay mixture contained 25 mM Tris (pH 8), 2 µM pyridoxal-phosphate, 2 mM EGTA, 0.25 mg/ml BSA, 1 mM pyruvate and 5 mM DL-β-aminoisobutyrate or beta-alanine. Vigabatrin (0.5 mM) and AOA (2 µM) were added to the activity assay and the reaction was started by the addition of 30 µl HEK293T cell extracts and left to proceed for 4 h at 37°C before stopping (5 min at 80°C). Appropriate blanks in the absence of DL-β-aminoisobutyrate or beta-alanine were also run in parallel. In the second step, the L-alanine produced was quantified in an end-point assay performed in 0.8 ml mixture containing 0.15 ml of the first reaction mixture in freshly prepared 20 mM Tris/0.5 M hydrazine buffer (pH 9), 0.7 mM EDTA and 0.9 mM NAD^+. The reaction was started by addition of 5 µl (2 U) of recombinant alanine dehydrogenase from *Bacillus cereus* (> 350 U/ml) and the change in absorbance at 340 nm was monitored for each sample.

Part 2: animal nutritional intervention study

Animal care and experimental protocol

A total of 66 male C57BL/6 mice (8 weeks old) were used in this study, divided over six groups. Upon arrival, mice were allowed to acclimatize to their new surrounding for 10 days before the start of the 2 week intervention period. All animals were allowed free access to food (standard chow not containing carnosine or derivatives) and water at room temperature and were exposed to a 12:12 h light/dark cycle.

Mice were randomly divided in groups and underwent different treatments (Table 1). Mice received different drinks depending on the amount of beta-alanine dissolved in the drinking water (ranging from 0, 0.1, 0.6 and 1.2% w/v). Mice from the 0.1% beta-alanine supplementation group were further divided in subgroups based on daily subcutaneous injections with beta-alanine transaminase inhibitors: vigabatrin, AOA or saline (SAL) as a control. Vigabatrin (Sabril; Lundbeck, Deerfield, IL, USA) was administered at a dose of 500mg/kg body weight in aqueous solution (50mg vigabatrin/ml saline or 10µL injection volume/g body weight). AOA (Sigma, St Louis, MO, USA) was administered in a dose of 10mg/kg body weight in aqueous solution (1mg AOA/ml saline or 10µL injection volume/g body weight). The same injection volume was used for saline. Drinking water was

refreshed at least three times a week and body weight and drinking volume per cage (two or three animals) were monitored.

The last inhibitor injection was performed 3 h prior to dissection. Mice were anaesthetized by an intraperitoneal infusion of 80% xylazine – 20% Ketamine (5 µL/g body weight). After careful dissection of soleus, tibialis anterior and gastrocnemius muscles and blood collection by cardiac puncture, mice were killed by cervical dislocation. Kidneys, liver, heart and brain were dissected and urine was collected from the bladder. Any visible connective or fat tissue was removed from the tissues and all samples were quickly frozen in liquid nitrogen and stored at -80°C. Blood samples were centrifuged at 16,000 g for 5 min at 4°C and serum was stored at -80°C.

Table 1. Characteristics of mice of the nutritional intervention study (Part 2)

%BA in DW	Injection solution	n	BW start (g)	Δ BW (g)	Drinking volume (ml/mouse/day)	Total dose (g BA/mouse)
0%	Saline	15	27 ± 1.5	1.5 ± 0.8	6.34 ± 1.24	/
0.1%	Saline	15	25 ± 1.9 §	1.3 ± 1.2	4.91 ± 0.79 §	0.069 ± 0.011
	Vigabatrin	15	25.5 ± 2.3	-0.4 ± 0.9 *	4.39 ± 0.51 *	0.061 ± 0.007
	AOA	8	26.8 ± 1.9	-0.5 ± 0.9 *	5.76 ± 0.14 *	0.081 ± 0.002
0.6%	Saline	7	26.3 ± 3.2	1.5 ± 0.8	5.25 ± 0.35 §	0.441 ± 0.030
1.2%	Saline	6	26.7 ± 1.5	1.6 ± 1.4	4.15 ± 0.99 §§	0.776 ± 0.108

§ $p < 0.05$ and §§ $p < 0.001$ vs 0% beta-alanine – SAL and * $p < 0.05$ vs 0.1% beta-alanine – SAL. Data are means ± SD. BA, beta-alanine; BW, body weight; DW, drinking water.

Preparation of mouse liver extracts

Mouse liver extracts (n=3) were prepared by homogenizing frozen samples in 3 volumes (w/v) of extraction buffer (50 mM potassium phosphate buffer, pH 7, 0.1% Triton X-100, 25 µM pyridoxal-phosphate and 5 µg/ml of leupeptine and antipaine), followed by centrifugation (16,000 g for 20 min at 4°C) and collection of the supernatant containing soluble proteins. Liver extracts were stored at -80°C.

Assays of GABA-T and AGXT2 transaminase activities

GABA-T and AGXT2 activity was measured as described above, except the reaction was started by addition of mouse liver extract.

mRNA expression of carnosine-related enzymes and transporters in mouse tissues by means of quantitative PCR

Total RNA from mouse skeletal muscles, heart, liver, kidney and brain was isolated using the TriPure Isolation Reagent (Roche, Basel, Switzerland) followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Walthem, MA, USA) and RNA purity was assessed using the A_{260}/A_{280} ratio. Using a blend of oligo(dT) and random primers, 500 ng of RNA was reversed transcribed with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in accordance with to the manufacturer's instructions. Quantitative PCR was carried out on a Lightcycler 480 system (Roche) using an 8 µL reaction mix containing 3 µL of template cDNA (1:10 dilution), 300 nM forward and reverse primers and 4 µL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions comprised a polymerase activation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Primer sequences (Table 2) of most genes of interest (CARNS, taurine transporter (TauT) and GABA-T) are available in the literature (Everaert *et al.*, 2013*a*). The primer sequence for AGXT2 was newly designed using Primer Express 3.0 (Applied Biosystems). Sequence specificity was confirmed using NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To control the specificity of amplification, data melting curves were inspected and PCR efficiency was calculated for AGXT2. Normalized gene expression values were calculated by dividing the relative gene expression values (calculated by the ΔCt method) for each sample by the expression values of the geometric mean of Ppia, Rplp0 and GAPDH as selected by GeNorm (Vandesompele et al., 2002).

Quantification of beta-alanine and GABA by means of high-performance liquid chromatography

Brains were dissolved in PBS (1 mg wet weight brain/10 µL) for homogenization. Brain homogenates, serum and urine were deproteinized using 35 % sulfosalicylic acid and centrifuged (5 min, 16,000 g). Deproteinized supernatant was mixed with AccQ Fluor Borate buffer and reconstituted Fluor Reagent (1:7:2) from the AccQTag chemistry kit (Waters, Milford, MA, USA). The same method was applied to the combined standard

solutions of beta-alanine (Sigma) and GABA (Sigma). The derivatized samples were applied to a Waters high-performance liquid chromatography system comprised of an AccQTag column (3.9 x 150 mm, 4 µm) and fluorescence detector (excitation/emission wavelength: 250/395 nm). The column was equilibrated with buffer A [10% eluent A (Waters) – 90% H2O], buffer B (100% acetonitrile) and buffer C (100% H_2O) at a flow rate of 1ml/min at room temperature. Urinary beta-alanine values were normalized to creatinine using the creatinine assay kit (Sigma).

Quantification of histidine-containing dipeptides by liquid chromatographyC-mass spectrometry

Tissue carnosine and anserine levels were measured using a Micromass ZMD mass spectrometer (Waters) (Baba et al., 2013). Homocarnosine was also measured in the brain. Briefly, the tissues were homogenized in PBS buffer containing the protease inhibitor (1:100) and internal standard tyrosine-histidine. The homogenates were centrifuged at 16,000 g for 10 min. The pellets were discarded and the supernatant was precipitated by perchloric acid. The samples were neutralized by ammonium hydroxide and diluted in 90% H_2O, 10% acetonitrile, 0.1% heptafluorobutyric acid. The peptides were separated by reverse phase elution with a polar RP column protected by a polar RP guard column. The solution was infused into the mass spectrometer in the positive ion mode. The spectrometer was calibrated using NaCsl with the calibration routine included in MassLynx, version 3.4 (Waters). Samples were diluted in 70%water:30%acetonitrile and the solution was infused using a glass syringe and a Harvard infusion pump at a rate of 10 µl/min. Tuning conditions were capillary 2.9kV, cone 34V, extractor 9V, Rflens 0.9V, source temperature 100°C, desolvation gas 200C, low mass resolution 15.2, ion energy 0.3V multiplier 650 relative setting. The acquisitons for carnosine (parent ion 227Da, daughter ion 110Da), anserine (parent ion 241Da, daughter ion 109.2Da), homocarnosine (parent ion 241Da, daughter ion 109.22Da), and tyrosine histidine (parent ion 319Da and daughter ion 110.22Da) were taken in the multiple reaction monitoring mode. The limits of detection for carnosine and anserine are 0.00367nmol and 0.0303nmol, respectively. The limits of quantification for carnosine and anserine is 0.011nmol and 0.0917nmol, respectively.

Table 2. Primers used in quantitative PCR analysis

Function	Gene symbol	Forward primer (5'-3') Reverse primer (5'-3')	Source
Carnosine synthesis	CARNS	TGA-TAG-GCC-CCT-ACT-GAG-TAA-GGT TCA-GTG-TCC-TTG-GCA-GGG-TAT	Everaert et al., 2013
Beta-alanine transport	TauT	TGG-CCG-ACA-GCA-TTC-CA GCC-TTC-TCT-AAG-GTG-CCT-TCC-T	Everaert et al., 2013
Beta-alanine transaminase	GABA-T	CCT-TCA-TGG-GTG-CTT-TCC-A CAA-AGG-AAG-GGA-TGT-CAA-TCT-TG	Everaert et al., 2013
	AGXT2	GAT-AGG-CTG-CCA-ATC-AAC-AAT-GT TGC-ACT-GGA-GAA-TCT-CGA-CAA	Primer express
Reference genes	Ppia	CAA-ATG-CTG-GAC-CAA-ACA-CAA-ACG GTT-CAT-GCC-TTC-TTT-CAC-CTT-CCC	RTprimerDB
	Rplp0	GGA-CCC-GAG-AAG-ACC-TCC-TT GCA-CAT-CAC-TCA-GAA-TTT-CAA-TGG	RTprimerDB
	GAPDH	CAC-CAT-CTT-CCA-GGA-GCG-AG CCT-TCT-CCA-TGG-TGG-TGA-AGA-C	RTprimerDB

GABA-T activity in brains

GABA-T activity was measured in brain samples according to the method of Awad et al. (2007). Brain samples were stored at -80°C until homogenization in 10 volumes of chilled buffer of the composition: 20% glycerol, 0.13% Triton X-100, 0.1 mM glutathione, 1 mM Na_2EDTA, 10 mM K_2HPO_4, 0.1 mM Pyroxidal-5'-phosphate and acetic acid to bring the pH to 6.8. The homogenates were frozen and thawed once before adding to the incubation medium (20µL) on a 96-well plate. The incubation medium (180µL) consisted of 100 mM potassium pyrophosphate, 3.5 mM 2-mercaptoethanol, 0.01 mM pyroxidal-5'-phosphate, 5 mM 2-oxoglutarate, 4 mM NAD^+. The samples were pre-incubated for 15 min at 37°C before the addition of GABA (10mM final concentration). The rate of the enzymatic reaction was determined by measuring NADH production at 37°C for 10 min within the linear range, using a i-control (infinite 200Pro) spectrophotometer (Tecan, Männedorf, Switzerland) (excitation/emission wavelength: 360/465 nm). Enzymatic activity was calculated relative to a control sample (distilled H_2O instead of GABA) using a NADH standard curve.

Statistics

Data are reported as the mean ± SD. $P \leq 0.05$ was considered statistically significant. The body weight at start, change in body weight and drinking volume were evaluated separately for the 4 oral beta-alanine supplementation doses on the one hand and the 0.1% beta-alanine groups treated with different inhibitors on the other hand. One-way analysis of variance (ANOVA) followed by a post hoc Tukey's test in the case of a significant group effect was used. A general linear model repeated measures ANOVA was used to evaluate body weight over time (start to end). The dose-response effect of different dosages of beta-alanine supplementation on HCD loading and the effect of treatments with different inhibitors on enzyme activity and tissue metabolites was evaluated by one-way ANOVA followed by a post hoc Tukey's test in the case of a significant group effect. For the urine and blood parameters, an independent sample t test was used to evaluate the effect of 0.1% beta-alanine supplementation compared to 0% beta-alanine and a one-way ANOVA and, subsequently, a post hoc Tukey test was used to evaluate the effect of treatments with different inhibitors compared to the 0.1% beta-alanine group. Correlations between serum beta-alanine levels and tissue HCD content were obtained by means of Pearson

correlations. All statistical analyses were performed using SPSS, version 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Activity of recombinant mouse GABA-T and AGXT2 in HEK293T cell extracts and effect of vigabatrin and AOA

The enzymatic activity of GABA-T was detected in transfected cell-extracts and was similar in the presence of 2.5 mM GABA and beta-alanine (Fig 1A), confirming that *in vitro* beta-alanine was a good substrate for GABA-T. In this case, the activity measured in the presence of 2.5 mM GABA was decreased by 75% and 86% when 0.5 mM vigabatrin or 2 µM AOA, respectively, was added to the assay (Fig 1C). Similarly, cell-extracts containing recombinant AGXT2 showed enzymatic activity in the presence of 5 mM beta-alanine and DL-β-aminoisobutyrate (Fig 1B) but, in contrast to GABA-T, AGXT2 activity was only inhibited by AOA (95% inhibition) and not by vigabatrin (Fig 1D). Similar results were obtained with recombinant human GABA-T and AGXT2 (data not shown).

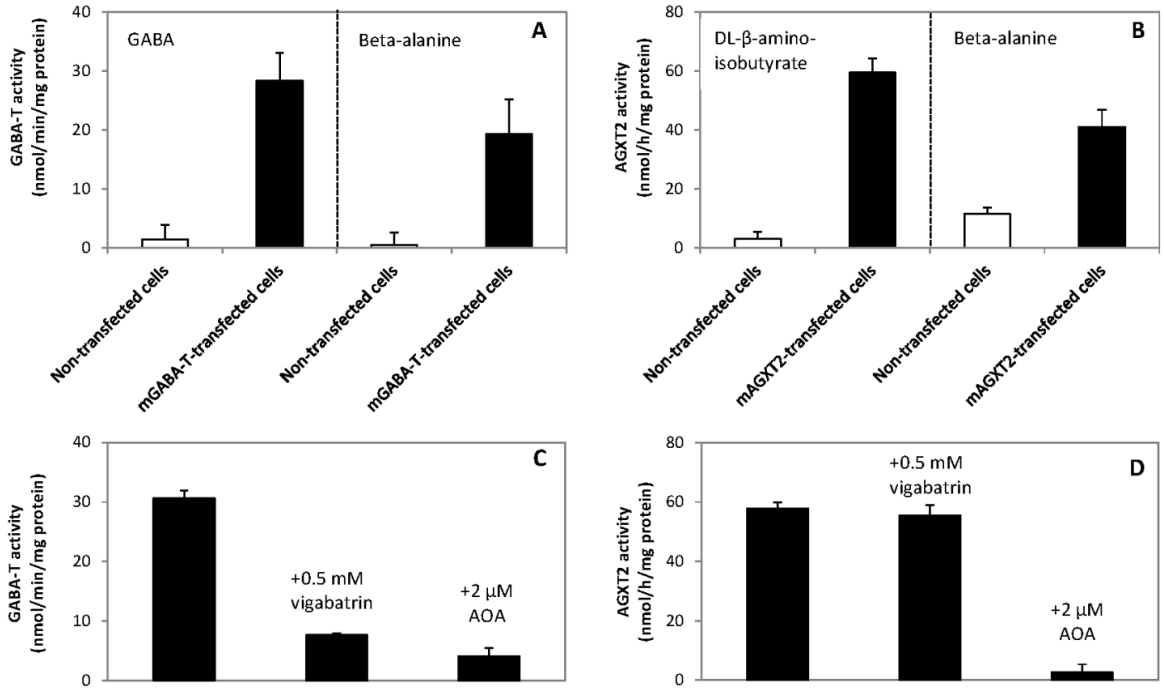

Figure 1. Activity of recombinant mouse GABA-T and AGXT2 (mGABA-T and mAGXT2) in cell extracts of HEK293T and inhibition by vigabatrin and aminooxyacetate (AOA). HEK293T cells were transfected or not with plasmids expressing mouse GABA-T (*A* and *C*) or AGXT2 (*B* and *D*). The enzymatic activities of recombinant GABA-T and AGXT2 were assayed *in vitro*, using the corresponding cell extracts, in the presence of 2.5 mM GABA and 5 mM DL-beta-aminoisobutyrate, respectively (*A* and *B*). The effect of vigabatrin (0.5 mM) and AOA (2 µM) on the enzymatic activities was tested by adding the inhibitors directly to the assay mixture as described in Methods (*C* and *D*). Values are the mean ± SD of three independent measurements.

mRNA expression profiles of carnosine-related enzymes and transporters in different mice tissues

The taurine transporter (TauT), responsible for transmembrane transport of beta-alanine, was ubiquitously expressed in all murine tissues investigated. CARNS, which is the enzyme responsible for carnosine and homocarnosine synthesis, was expressed mainly in the brain and the striated muscles, with highest mRNA levels in glycolytic (gastrocnemius and tibialis anterior muscles) rather than oxidative muscles (soleus, heart) (Fig 2A&B). The beta-alanine transaminating enzymes GABA-T and AGXT2 showed highest mRNA expression in the liver and kidney, low mRNA expression in oxidative muscles, and even lower expression in glycolytic muscles. However, GABA-T, but not AGXT2, was also clearly expressed in the brain (Fig 2C&D).

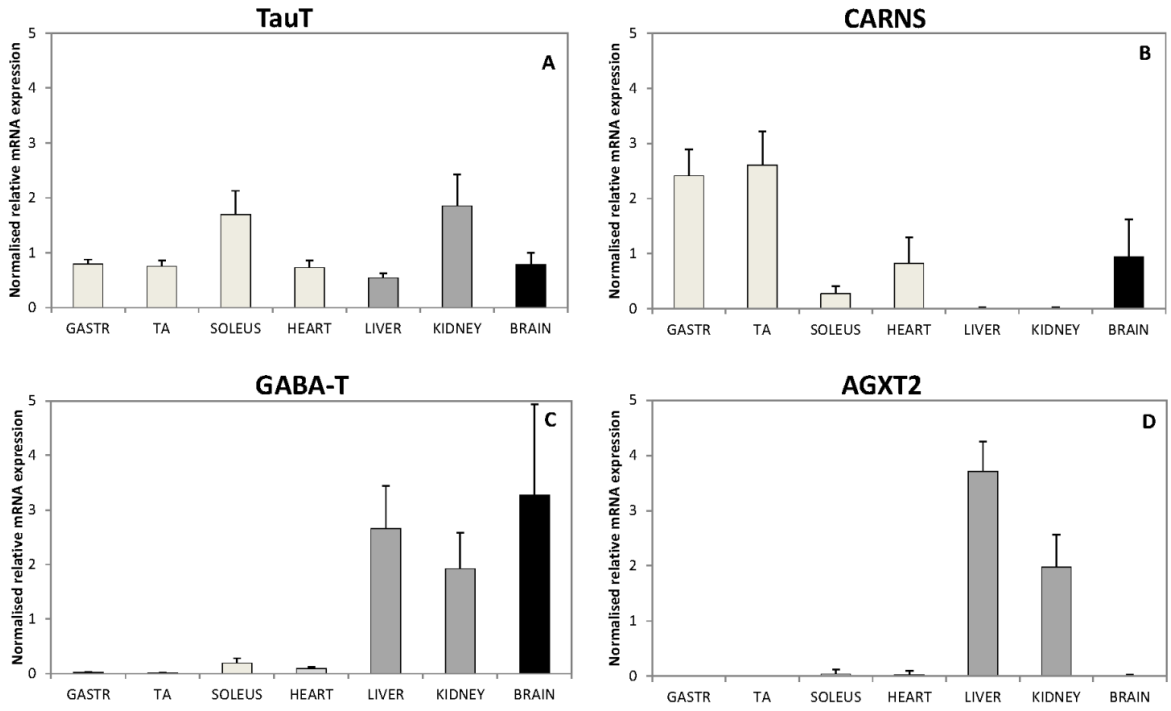

Figure 2. mRNA expression profile of carnosine-related enzymes and transporters in different mice tissues (three skeletal muscles, heart, liver, kidney and brain). mRNA expression profile is shown for TauT (panel A), CARNS (panel B), GABA-T (panel C) and AGXT2 (panel D). GASTR, gastrocnemius; TA, tibialis anterior. Values are the mean ± SD of six measurements for each tissue.

Effect of vigabatrin and AOA on GABA-T and AGXT2 activity in liver extracts

Because both GABA-T and AGXT2 were highly expressed in liver, we tested the effects of *in vivo* administration of vigabatrin and AOA on the *ex vivo* activity of these enzymes in liver extracts. In agreement with the results found for the recombinant enzymes (Fig 1),

the administration of AOA resulted in the strong inhibition of both GABA-T (-83%) and AGXT2 (-99%) enzymatic activities, whereas vigabatrin was a more specific inhibitor of GABA-T activity (-81%) and only minimally affected the activity of AGXT2 (-26%) (Fig 3A&B).

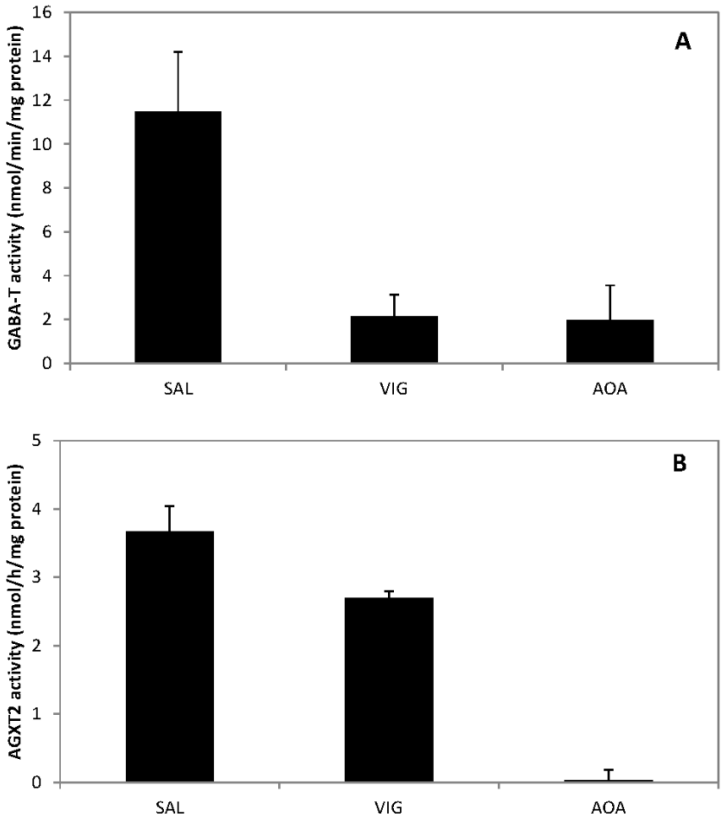

Figure 3. Effect of vigabatrin and AOA on GABA-T and AGXT2 activity in liver extracts.

Effect of administration of vigabatrin or AOA on the ex vivo activity of beta-alanine transaminating enzymes (GABA-T and AGXT2) measured in liver extracts from mice supplemented with 0.1% beta-alanine. GABA-T activity (A) was measured with 2.5 mM GABA and 1 mM α-ketoglutarate and AGXT2 (B) with 5 mM DL-beta-aminoisobutyrate and 1 mM pyruvate. The effect of vigabatrin and AOA on the liver enzymatic activities is the result of the 14 day treatment that the mice were subjected to (prior to the preparation of the extract) and not to the addition of the inhibitors directly to the assay mixture, as described in the Methods. VIG, vigabatrin. Values are the mean ± SD of three independent measurements.

Dose-response relationship of beta-alanine supplementation

Oral beta-alanine supplementation (0.1% beta-alanine w/v in drinking water for 2 weeks) had no effect on the HCD storage in either gastrocnemius or the soleus muscle (Fig 4A&B). However, supraphysiological doses of 0.6% and 1.2% of beta-alanine led to significant increases in total muscle HCD content. In soleus muscle, which is characterized by low amounts of carnosine and anserine, 3- to 4-fold increases were found ($p \leq 0.001$ for 0.6%

and 1.2% compared to 0% beta-alanine). In gastrocnemius, a 40-50% increase in the levels of HCDs was found in mice supplemented with 0.6% and 1.2% beta-alanine (p = 0.004 and p = 0.017 compared with 0% beta-alanine). The lack of increase in muscle HCD concentrations with 0.1% beta-alanine suggests that, at these levels of oral supplementation, all the ingested beta-alanine is transaminated. Subsequent inhibitor experiments were therefore performed in mice supplemented with this dose of beta-alanine.

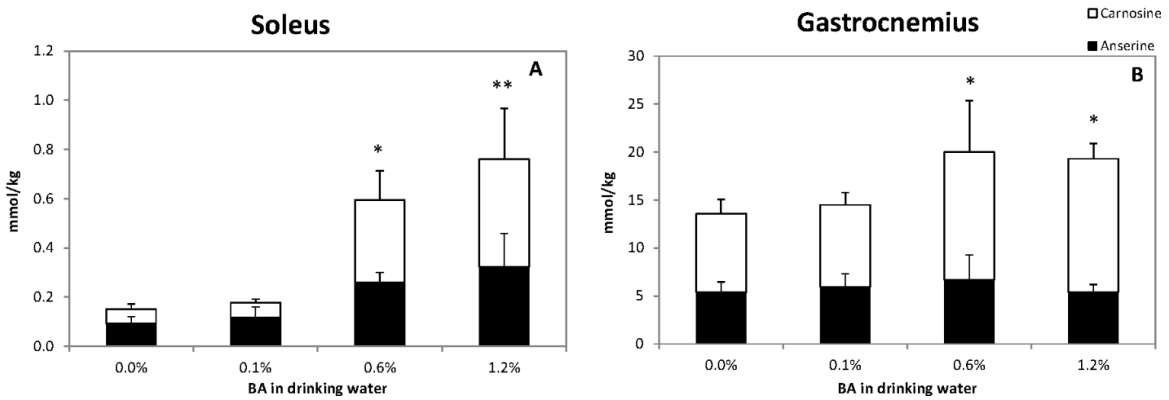

Figure 4. Dose-response relationship of beta-alanine supplementation in soleus and gastrocnemius.
Dose–response relationship between the amount (w/v) of beta-alanine (BA) added to the drinking water (0%, 0.1%, 0.6% or 1.2%) and the HCD levels for musculus soleus (A) and musculus gastrocnemius (B). ∗P < 0.05 and ∗∗P < 0.001 vs. 0% beta-alanine – SAL. Significant differences refer to total HCD content (sum of carnosine and anserine). Values are the mean ± SD.

Effect of inhibitors on serum and urinary beta-alanine levels

Although there was no significant effect of 0.1% beta-alanine supplementation by itself, serum and urinary beta-alanine levels were significantly increased when this low amount of oral beta-alanine was combined with AOA (+218%, p = 0.009 and +250%, p = 0.001 vs 0.1% beta-alanine – SAL for serum and urine, respectively). Vigabatrin did not significantly affect serum, nor urinary beta-alanine levels (+84%, p = 0.486 and +4.0%, p = 0.997 vs 0.1% beta-alanine – SAL for serum and urine, respectively) (Fig 5A&B). Individual data points are shown in figure 5 to demonstrate the large inter-individual variation that was observed.

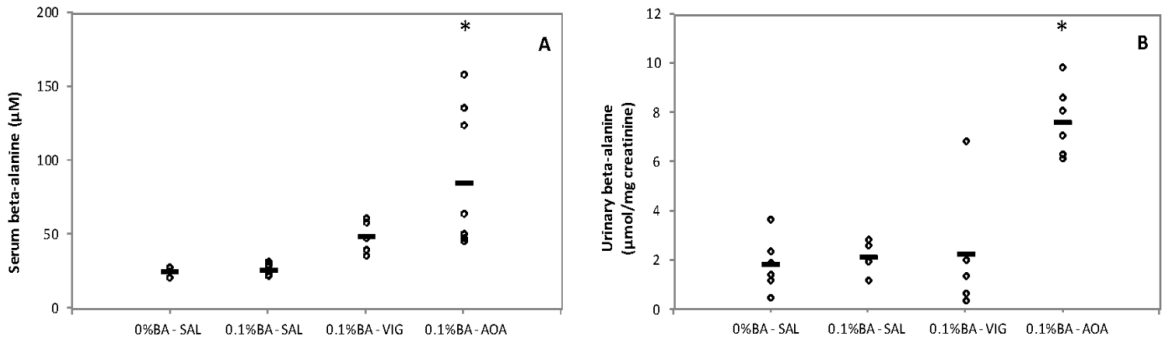

Figure 5. Effect of inhibitors on serum and urinary beta-alanine levels.

Effect of oral 0.1% beta-alanine (BA) supplementation combined with SAL, vigabatrin (VIG) or AOA, on serum (A) and urinary (B) beta-alanine levels. ∗P < 0.05 vs. 0.1%beta-alanine – SAL. Symbols represent individual results (circles) and the mean (dash).

Effect of inhibitors on tissue HCD levels

Supplementation of 0.1% beta-alanine did not affect the HCD levels in any of the investigated tissues. However, AOA coadministration led to significantly higher HCD loading in the different muscles (Fig 6A-D). The highest effects were found in the soleus muscle and the heart, with increases of 128% and 541%, respectively ($p < 0.001$ vs 0.1% beta-alanine – SAL). In soleus and heart, but not in the more glycolytic muscles, serum beta-alanine was positively correlated with HCD levels ($r = 0.537$, $p = 0.008$ for soleus, $r = 0.570$, $p = 0.007$ for heart). The more glycolytic muscles also showed significantly (or trending to significance) higher HCD loading with AOA administration (+105%, $p = 0.011$ and +21%, $p = 0.056$ vs 0.1% beta-alanine – SAL for tibialis anterior and gastrocnemius, respectively), although the effects were absent in kidney and brain (Fig 6E&F). Furthermore, no effects of vigabatrin on carnosine and anserine levels were found in any of the investigated tissues.

Brain GABA-T activity, brain GABA and homocarnosine levels and serum GABA

The brain GABA-T activity was significantly decreased with administration of vigabatrin or AOA (-82% and -88%, respectively; $p < 0.001$) (Fig 7A). Beta-alanine supplementation did not significantly affect brain GABA levels but led to a significant decrease in brain homocarnosine levels (-29%, $p = 0.05$ vs 0% beta-alanine – SAL). With vigabatrin and AOA administration, both brain GABA and homocarnosine levels were significantly increased (brain GABA: $p < 0.001$ vs 0.1% beta-alanine – SAL for vigabatrin and AOA, brain homocarnosine: $p = 0.015$ and $p = 0.027$ vs 0.1% beta-alanine – SAL for vigabatrin and

AOA, respectively) (Fig 7B&C). Serum GABA was not affected by beta-alanine supplementation and was only significantly elevated when vigabatrin was administered (+369%, p = 0.002 vs 0.1% beta-alanine - SAL), whereas AOA administration had no effect (Fig 7D).

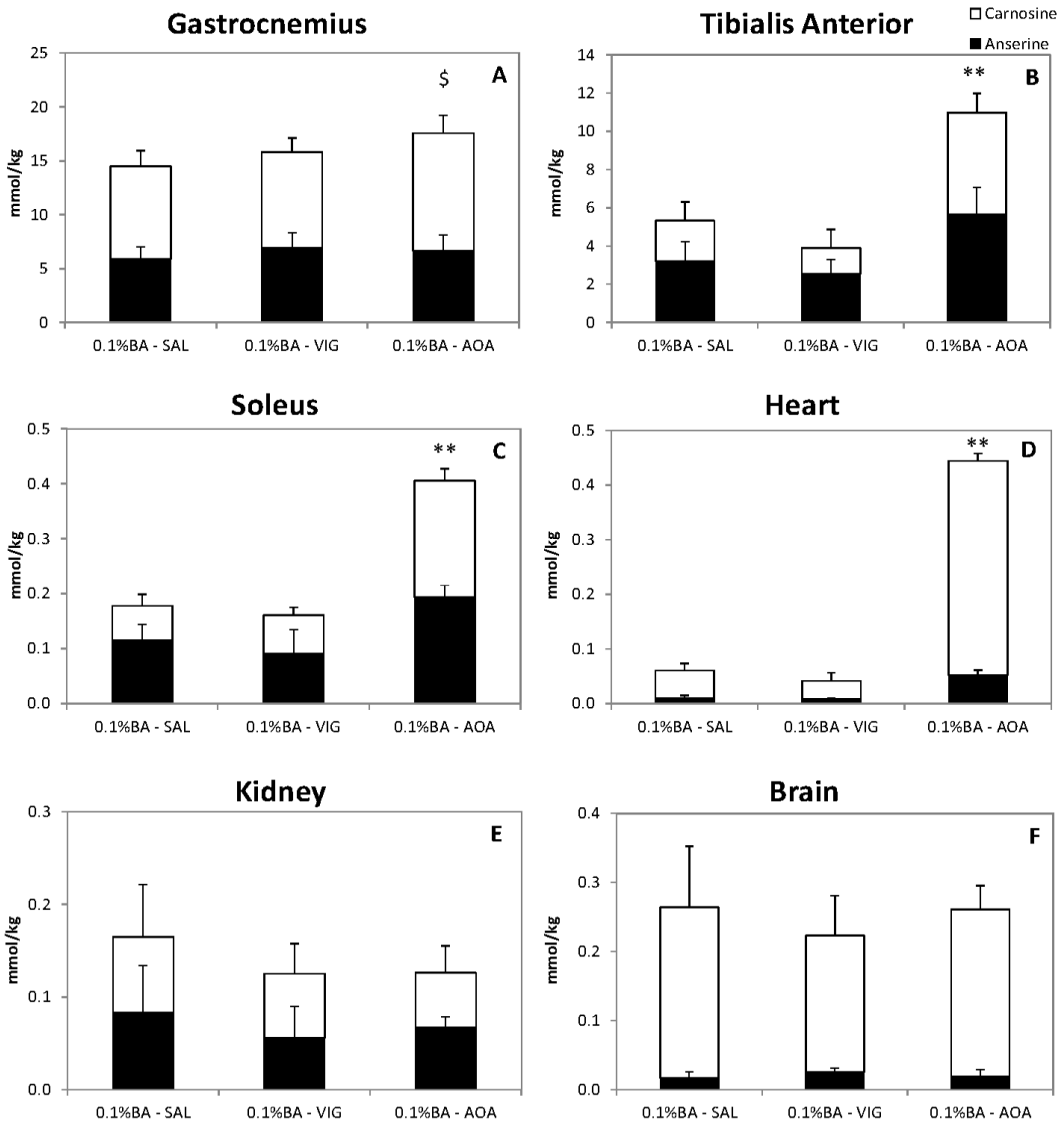

Figure 6. Effect of 0.1% beta-alanine (BA) supplementation combined with SAL, vigabatrin (VIG) or AOA on tissue HCD levels. HCD levels are shown for gastrocnemius and tibialis anterior (panel A-B), soleus and heart (panel C-D), kidney (panel E) and brain (panel F). ∗∗P < 0.001; $P > 0.05 and < 0.1 vs. 0.1%beta-alanine – SAL. Significant differences refer to total HCD content (sum of carnosine and anserine). Values are the mean ± SD.

Body weight and drinking behavior

Body weight at the start of the intervention was similar between groups, except for the 0.1% beta-alanine – SAL group, which showed a significantly lower body weight compared to the control group (p = 0.044, Table 1). Change in body weight was not significantly

different between groups supplemented with different amounts of beta-alanine, although their body weight over time did show a significant increase compared to the body weight at the start ($p < 0.05$ for all 4 groups). By contrast, change in body weight was different between the 0.1% supplemented groups treated with different inhibitors. Body weight gain over time was significantly lower in vigabatrin and AOA treated groups compared to 0.1% beta-alanine – SAL group ($p < 0.001$ and $p = 0.002$, respectively). In addition, the daily drinking volume of all beta-alanine supplemented groups was significantly lower compared to the control group ($p < 0.05$ for 0.1% and 0.6% beta-alanine and $p < 0.001$ for 1.2% beta-alanine vs 0% beta-alanine, Table 1). Mice treated with vigabatrin drank significantly less and mice treated with AOA drank significantly more than the 0.1% beta-alanine – SAL group ($p = 0.05$ and $p = 0.007$, respectively).

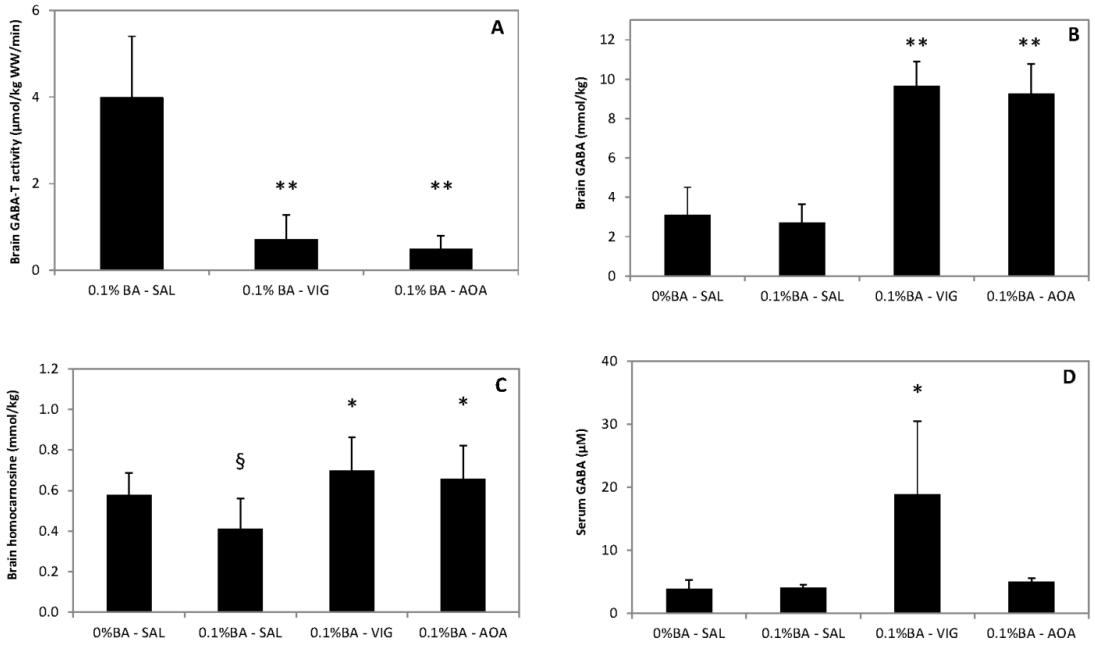

Figure 7. Effect of 0.1% beta-alanine (BA) combined with SAL, vigabatrin (VIG) or AOA on brain GABA-T activity (A) and concentrations of brain GABA (B), brain homocarnosine (C) and serum GABA (D). ∗P < 0.05 and ∗∗P < 0.001 vs. 0.1%beta-alanine – SAL; §P ≤ 0.05 vs. 0%beta-alanine – SAL. Values are the mean ± SD.

DISCUSSION

The present study aimed to clarify the contribution of beta-alanine degradation by GABA-T and/or AGXT2 to the metabolism and the homeostasis of HCDs in various mice tissues. Selective GABA-T inhibition by vigabatrin caused a moderate, non-significant elevation of circulating beta-alanine concentrations, indicating that GABA-T is probably involved in the degradation of beta-alanine upon oral ingestion in mice. Nevertheless, the rise in plasma beta-alanine was not sufficient to elevate HCD content in tissues that express CARNS (i.e. skeletal muscles and heart). Administration of AOA, which inhibits GABA-T to the same degree as vigabatrin and additionally inhibits AGXT2, resulted in much larger circulating and urinary beta-alanine levels. In turn, this had a marked positive effect on tissue HCD content. This effect was most pronounced in oxidative-type striated muscles (soleus and heart), less pronounced in glycolytic-type muscles (gastrocnemius and tibialis anterior) and absent in the kidney or the brain. The overview of our current understanding of the beta-alanine metabolism, based on the results of the present study, is illustrated in Fig 8.

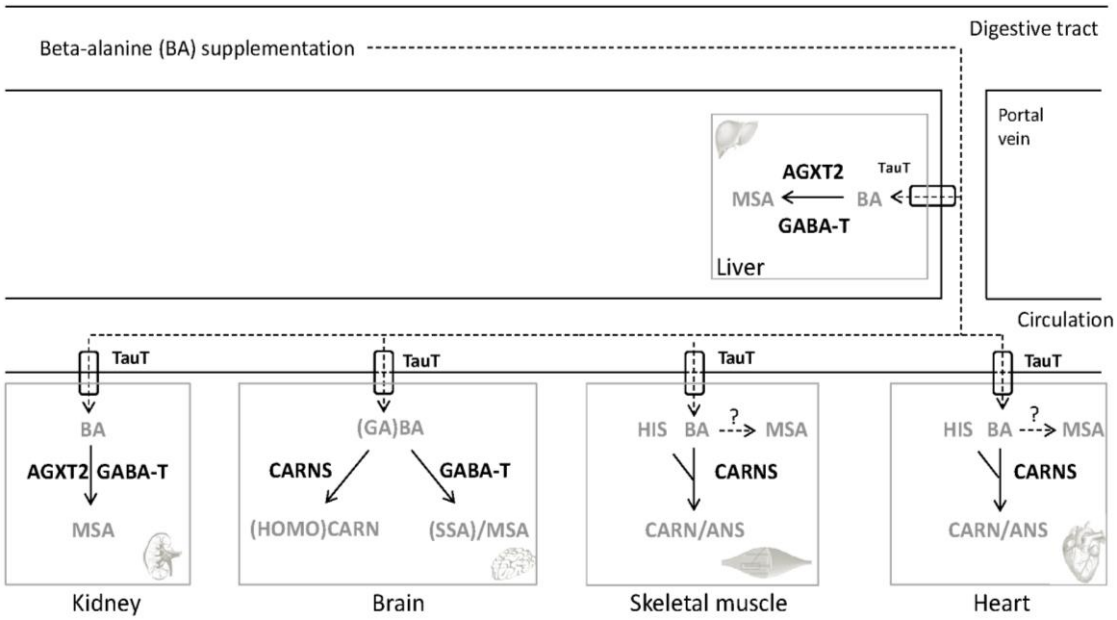

Figure 8. Overview of the current understanding of the beta-alanine metabolism. Beta-alanine transaminating enzymes GABA-T and AGXT2 are expressed at mRNA level in liver and kidney, converting beta-alanine to MSA. CARNS is mainly expressed in the brain, skeletal muscles and heart. Inhibiting GABA-T and AGXT2 leads to increased circulating levels of beta-alanine, which can be taken up in different tissues by the TauT and subsequently converted to carnosine and anserine. ANS, anserine; BA, beta-alanine; (HOMO)CARN, (homo)carnosine; HIS, histidine; SSA, succinate semi-aldehyde.

It is currently assumed that beta-alanine is transaminated into MSA mainly by GABA-T and AGXT2. Enzymatic assays have already indirectly demonstrated that these enzymes can transaminate beta-alanine in liver, kidney and brain extracts (Tamaki et al., 1990; Kontani et al., 1998; Ito et al., 2001), but the current study provides the first strong direct evidence using pure proteins obtained by recombinant DNA technology (Fig 1A&B). Furthermore, using this approach, we could confirm that GABA-T, but not AGXT2, is inhibited by vigabatrin and that both these enzymes are inhibited by AOA (Fig 1C&D). Although, on the basis of these data, we cannot exclude the involvement of other enzymes in this process, to our knowledge, there are no other known mammalian enzymes that can transaminate beta-alanine. The finding that circulating levels of beta-alanine were markedly affected when both GABA-T and AGXT2 were inhibited *in vivo*, further supports this notion.

Gene expression of beta-alanine transaminating enzymes is shown to be high in liver and kidney and GABA-T is, unlike AGXT2, also expressed in the brain of control mice (Fig 2C&D). In line with the *in vitro* results in transfected HEK293t cells, *ex vivo* GABA-T and AGXT2 activity in liver extracts was affected in the same way following *in vivo* administration of vigabatrin and AOA (Fig 3A&B). Specific GABA-T inhibition by vigabatrin, however, did not significantly affect circulating beta-alanine levels whereas inhibition of both enzymes by AOA led to a marked increase in these levels *in vivo* (Fig 5A), suggesting that the combined inhibition of both these enzyme increases the circulating levels of this amino acid.

To demonstrate that the transamination of beta-alanine is involved in HCD homeostasis, we searched for a suitable oral beta-alanine dose that did not increase muscle HCD content on its own, meaning that all exogenously provided beta-alanine is fully routed toward oxidation rather than dipeptide synthesis. Of the three different doses tested, we found that muscle HCD levels remained stable only with the lowest dose (0.1% beta-alanine), whereas both 0.6% and 1.2% beta-alanine increased HCD content above the physiological set point, suggesting that the beta-alanine transaminases (GABA-T and AGXT2) are saturated and no longer able to metabolize all circulating beta-alanine (= i.e., fail to avoid a rise in circulating beta-alanine) (Fig 4A&B). Hence, to ensure that the beta-

alanine transaminases are not saturated, we performed all subsequent *in vivo* experiments with the lowest dose (0.1% beta-alanine).

In accordance with the results showing that circulating beta-alanine levels were increased only upon inhibition of both transaminase enzymes, only AOA administration led to significantly elevated HCD levels in gastrocnemius, tibialis anterior, soleus and heart (Fig 6A-D). This effect was absent in kidney, presumably because CARNS is not highly expressed in this tissue (as assumed from the mRNA expression profile). However, specific GABA-T inhibition by vigabatrin did not have an effect on the HCD content in any of the tissues. Because we did not have an inhibitor specific for AGXT2, which does not act on GABA-T, the contribution of GABA-T to the beta-alanine metabolism remains unclear. However, our results are consistent with the idea that either GABA-T does not play an important role in the transamination process or that AGXT2 is able to compensate for the loss in GABA-T activity when vigabatrin is administered. Nevertheless, when taken together, these data clearly demonstrate that beta-alanine transaminases are involved in tissue HCD homeostasis.

As noted above, we found that both GABA-T and AGXT2 are highly expressed in liver and kidney, but show very low mRNA expression in the different muscles. However, the *in vivo* effects of AOA were most pronounced in oxidative-type muscles (soleus and heart) and less pronounced in glycolytic-type muscles (tibialis anterior and gastrocnemius). Because GABA-T and AGXT2 are mitochondrial enzymes and oxidative muscles have more mitochondria compared to glycolytic muscles, it is reasonable to propose that, in addition to oxidation in liver and kidney, beta-alanine can also be locally oxidized in these muscle cells. Figure 2C&D show the low but detectable mRNA expression of GABA-T and AGXT2 in the soleus and the heart, but not in the tibialis anterior and gastrocnemius muscles. Hence, even though it appears that beta-alanine oxidation takes place mainly in liver and kidney, additional peripheral beta-alanine degradation could possibly take place in oxidative muscles. Using radioactive labelled beta-alanine, Tamaki et al. (1980) demonstrated that beta-alanine is more stable in rat gastrocnemius (half-life of 2.27hr) compared to liver (half-life of 0.41hr), but still much less stable than muscle carnosine (half-life of 28 days), suggesting that the peripheral degradation of beta-alanine is possible. This possibility is further supported by a recent study (Hatazawa et al., 2015)

demonstrating that, in comparison with wild-type mice, GABA-T is upregulated (4.0-fold) in the muscles of transgenic mice overexpressing skeletal muscle specific peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α). Furthermore, muscle beta-alanine, carnosine and anserine levels were decreased in these transgenic mice (ranging from 0.04 to 0.15-fold). These findings support the hypothesis that, in oxidative muscles, intramuscular beta-alanine is converted into acetyl-coenzyme A (via MSA) and subsequently enters the citric acid cycle.

By far the largest effect of beta-alanine transaminase inhibition was found in the heart, showing 10-fold higher HCD levels when 0.1% beta-alanine was administered simultaneously with the transaminase inhibitor AOA (Fig 6D). To our knowledge, this is the first study showing an intervention that can elevate HCD content in the heart. A recent study of Swietach et al. (2013) demonstrated that HCDs can act as diffusible Ca^{2+}/H^{+} exchangers in cardiomyocytes because both calcium ions and protons can competitively bind to proteins and dipeptides, such as the HCDs. Ca^{2+} signalling regulates many cell functions and is modulated by H^{+} ions, suggesting that spatial Ca^{2+}/H^{+} coupling is probably of general importance in cell signalling and function. An additional pathophysiological relevance of carnosine was recently reported by Baba et al. (2013), who demonstrated that carnosine plays an important role in detoxifying reactive aldehydes and promotes functional recovery in the ischemic heart. Taken together, these recent studies indicate an important role of HCDs in the heart and suggest that an increase in HCD levels could positively influence cardiac function and the resistance of the heart to ischemic injury.

In the present study, we found that, upon moderate dietary beta-alanine exposure, beta-alanine transaminases can degrade all excess exogenous beta-alanine to maintain tissue HCD homeostasis. Hence, to elevate muscle HCD content, it may be necessary to first saturate these enzymes to achieve significant HCD loading. This condition is probably not met under normal human dietary situations. However, the selective ingestion of beta-alanine in high doses, as in human athletes ingesting 4-6g of pure beta-alanine for several weeks, can saturate the transaminases and lead to elevated muscle HCD content. The activity of beta-alanine transaminating enzymes probabaly explains the low muscular uptake and loading efficiency (3-6%) of beta-alanine supplementation, as calculated by

Stegen et al. (2013). Because beta-alanine is first routed towards the degradation pathway, sufficient beta-alanine needs to be ingested, resulting in a rather inconvenient supplementation protocol. This is fortified by case reports in which enhanced beta-alanine and carnosine levels are present in plasma and muscles, respectively, which is suggested to be attributed to genetic deficiencies in the beta-alanine transaminase enzymes (Scriver et al., 1966; Jaeken et al., 1984). However, improving the efficiency of the beta-alanine supplementation protocol in healthy humans by inhibiting transaminases appears to be difficult because of the toxicity (AOA) and side-effects (vigabatrin) of these inhibitors and the multiple other metabolic pathways in which these transaminases are involved.

The metabolism of beta-alanine, as determined in the present study, closely resembles the metabolism of another non-proteinogenic amino acid, ɣ-aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter present in the brain and, similar to beta-alanine, a substrate for GABA-T. Interestingly, GABA is also a substrate for CARNS, synthesizing homocarnosine when combined with L-histidine. It has been shown that administration of vigabatrin results in a dose-dependent increase in GABA and homocarnosine levels in the brains of both rodents (Jung et al., 1977) and humans (Petroff *et al.*, 1998, 1999). In addition, AOA has similar effects on GABA levels in the brain (Wallach, 1961; Gelder, 1966; Löscher & Frey, 1978). In the present study, we confirm that GABA-T is expressed at mRNA level in the brain and inhibition by vigabatrin leads to increased GABA concentrations (Fig 7B), suggesting that GABA-T regulates GABA homeostasis. AGXT2 is not involved in this process. Brain carnosine and anserine were not affected by the administration of vigabatrin or AOA, probably as a result of the high GABA concentrations, with which beta-alanine has to compete to occupy CARNS. However, the metabolism of carnosine in the brain is not yet fully understood, although it probably does not depend upon the activity of hepatic transaminases.

The results of the present study show that mice treated with transaminase inhibitors (vigabatrin or AOA) have a reduced body weight gain during the intervention compared to mice treated with saline. This observation is in accordance with other studies (Howard et al., 1980; Gale & Iadarola, 1980) and is presumably related to disturbance of GABA homeostasis, which plays a role in the hypothalamic regulation of food intake.

The present study has some limitations. First, mRNA expression was used to estimate the protein expression and activity of the main enzymes in the different mice tissues. However, mRNA expression is not always in agreement with the amount of mRNA that is effectively translated into protein. Our data on mRNA expression should therefore be considered as an estimation for protein expression and function. However, when available, our expression data are in agreement with existing literature and probably provide qualitative information on the tissue distribution of the relevant enzymes. Second, two inhibitors were applied in this study: vigabatrin as a specific inhibitor for GABA-T and AOA as a non-specific inhibitor (inhibiting both GABA-T and AGXT2). Unfortunately, no specific inhibitor for AGXT2 was available. Therefore, we cannot compare the implications of inhibiting only AGXT2 vs inhibiting both enzymes on the metabolism of beta-alanine in mice.

In summary, the results of this study suggest that the homeostasis of the HCDs carnosine and anserine in cardiac myocytes and skeletal muscle is dependent on the circulating availability of beta-alanine. In turn, homeostasis of circulating beta-alanine is, in case of excess dietary beta-alanine intake, dependent on the degradation of beta-alanine in liver and kidney, which express GABA-T and AGXT2 as the main mammalian enzymes capable of metabolizing beta-alanine. The present study highlights the importance of beta-alanine transamination in tissue HCD homeostasis and thereby contributes to a better understanding of the mammalian beta-alanine and carnosine metabolism.

ACKNOWLEDGEMENTS

LB, JS, SS, WC, MVDC, WD and IE conceived and designed the experiments. LB, SPB, SK, JS, SB, WC, AAB, HJD, AB, JD, BV, MVDC, WD and IE collected, analysed and interpreted the data. LB, SPB, SK, JS, SS, SB, WC, AAB, HJD, AB, JD, BV, MVDC, WD and IE drafted the article and revised it critically for important intellectual content. All authors have read and approved the final submission. All authors agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Supported in part by a grant by the National Institutes of Health (HL122581) to SPB, as well as a grant from the Research Foundation – Flanders (FWO G.0243.11 and G.0352.13N) to WD. MVDC is Chercheur Qualifié of the Belgian Fonds National de la Recherche Scientifique (FRS-FNRS). LB is a recipient of a PhD Scholarship by Research Foundation – Flanders (Aspirant FWO). The technical assistance of A. Volkaert is greatly acknowledged.

REFERENCES

Awad R, Levac D, Cybulska P, Merali Z, Trudeau VL & Arnason JT (2007). Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. *Can J Physiol Pharmacol* **85,** 933–942.

Baba SP, Hoetker JD, Merchant M, Klein JB, Cai J, Barski O a, Conklin DJ & Bhatnagar A (2013). Role of aldose reductase in the metabolism and detoxification of carnosine-acrolein conjugates. *J Biol Chem* **288,** 28163–28179.

Baguet A, Reyngoudt H, Pottier A, Everaert I, Callens S, Achten E & Derave W (2009). Carnosine loading and washout in human skeletal muscles. *J Appl Physiol* **106,** 837–842.

Baxter C & Roberts E (1961). Elevation of y-aminobutyric acid in brain: Selective inhibition of y-aminobutyric-a-ketoglutaric acid transaminase. *J biol Chem* **236,** 3287.

Blancquaert L, Everaert I & Derave W (2015). Beta-alanine supplementation, muscle carnosine and exercise performance. *Curr Opin Clin Nutr Metab Care* **18,** 63–70.

Boldyrev AA, Aldini G & Derave W (2013). Physiology and pathophysiology of carnosine. *Physiol Rev* **93,** 1803–1845.

Derave W, Ozdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, Wise J a & Achten E (2007). Beta-alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *J Appl Physiol* **103,** 1736–1743.

Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V & Van Schaftingen E (2010). Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem* **285,** 9346–9356.

Dunnett M & Harris RC (1999). Influence of oral -alanine and L-histidine supplementation on the carnosine content of the gluteus medies. *Equine Vet J* **30,** 499–504.

Everaert I, Mooyaart A, Baguet A, Zutinic A, Baelde H, Achten E, Taes Y, De Heer E & Derave W (2011). Vegetarianism, female gender and increasing age, but not CNDP1 genotype, are associated with reduced muscle carnosine levels in humans. *Amino Acids* **40,** 1221–1229.

Everaert I, De Naeyer H, Taes Y & Derave W (2013). Gene expression of carnosine-related enzymes and transporters in skeletal muscle. *Eur J Appl Physiol* **113,** 1169–1179.

Gale K & Iadarola M (1980). Seizure protection and increased nerve-terminal GABA: delayed effects of GABA transaminase inhibition. *Science (80-)* **208,** 288–291.

Van Gelder N (1966). The effect of aminooxyacetic acid on the metabolism of γ-aminobutyric acid in brain. *Biochem Pharmacol* **15,** 533–539.

Harris RC, Dunnett M & Greenhaff PL (1998). Carnosine and taurine contents in individual fibres of human vastus lateralis muscle. *J Sports Sci* **16,** 639–643.

Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill C a, Sale C & Wise J a (2006). The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino Acids* **30,** 279–289.

Hatazawa Y, Senoo N, Tadaishi M, Ogawa Y, Ezaki O, Kamei Y & Miura S (2015). Metabolomic Analysis of the Skeletal Muscle of Mice Overexpressing PGC-1α. *PLoS One* **10,** e0129084.

Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK & Wise JA (2007). Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino Acids* **32,** 225–233.

Horváth V & Wanders R (1995). Aminooxy acetic acid: a selective inhibitor of alanine: glyoxylate aminotransferase and its use in the diagnosis of primary hyperoxaluria type I. *Clin Chim acta* **243,** 105–114.

Howard J, Cooper B, White H, Soroko F & Maxwell R (1980). A role for GABA in the control of ingestive behavior in rats: effects of ethanolamine-O-sulfate and muscimol. *Brain Res Bull* **5,** 595–599.

Ito S, Ohyama T, Kontani Y, Matsuda K, Fujimoto Sakata S & Tamaki N (2001). Influence of dietary protein levels on beta-alanine aminotransferase expression and activity in rats. *J Nutr Sci Vitaminol* **47,** 275–282.

Jaeken J, Casaer P & Cock P De (1984). Gamma-aminobutyric acid-transaminase deficiency: a newly recognized inborn error of neurotransmitter metabolism. *Neuropediatrics* **15,** 165–169.

John R, Charteris A & Fowler L (1978). The reaction of amino-oxyacetate with pyridoxal phosphate-dependent enzymes. *Biochem J* **171,** 771–779.

Jung M, Lippert B, Metcalf B, Bohlen P & Schechter P (1977). γ-vinyl GABA (4-amino-hex-5-enoic acid), a new selective irreversible inhibitor of GABA-T: effects on brain GABA metabolism in mice. *J Neurochem* **29,** 797–802.

Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT & Wise J a (2009). The effect of 4 weeks beta-alanine supplementation and isokinetic training on carnosine concentrations in type I and II human skeletal muscle fibres. *Eur J Appl Physiol* **106,** 131–138.

Kontani Y, Kawasaki S, Kaneko M, Matsuda K, Fujimoto Sakata S & Tamaki N (1998). Inhibitory effect of ethanol administration on beta-alanine-2-oxoglutarate

aminotransferase (GABA aminotransferase) in disulfiram-pretreated rats. *J Nutr Sci Vitaminol* **44,** 165–176.

Kurozumi Y, Abe T, Yao W & Ubuka T (1999). Experimental beta-alaninuria induced by (aminooxy) acetate. *Acta Med Okayama* **53,** 13–18.

Lippert B, Metcalf BW, Michel JJUNG & Casara P (1977). 4-Amino-hex-5-enoic Acid , a Selective Catalytic Inhibitor of 4-Aminobutyric-Acid Aminotransferase in Mammalian Brain. *Eur J Biochem* **74,** 441–445.

Löscher W & Frey H (1978). Aminooxyacetic acid: correlation between biochemical effects, anticonvulsant action and toxicity in mice. *Biochem Pharmacol* **27,** 103–108.

Petroff O a, Hyder F, Collins T, Mattson RH & Rothman DL (1999). Acute effects of vigabatrin on brain GABA and homocarnosine in patients with complex partial seizures. *Epilepsia* **40,** 958–964.

Petroff O a, Mattson RH, Behar KL, Hyder F & Rothman DL (1998). Vigabatrin increases human brain homocarnosine and improves seizure control. *Ann Neurol* **44,** 948–952.

Pihl A & Fritzson P (1955). The catabolism of C14-labeled β -alanine in the intact rat. *J Biol Chem* **215,** 345–351.

Rodionov RN, Jarzebska N, Weiss N & Lentz SR (2014). AGXT2: a promiscuous aminotransferase. *Trends Pharmacol Sci* **35,** 575–582.

Scriver C, Pueschel S & Davies E (1966). Hyper-β-alaninemia associated with β-aminoaciduria and γ-aminobutyricaciduria, somnolence and seizures. *N Engl J Med* **274,** 635–643.

Stegen S, Blancquaert L, Everaert I, Bex T, Taes Y, Calders P, Achten E & Derave W (2013). Meal and beta-alanine coingestion enhances muscle carnosine loading. *Med Sci Sports Exerc* **45,** 1478–1485.

Stout JR, Cramer JT, Zoeller RF, Torok D, Costa P, Hoffman JR, Harris RC & O'Kroy J (2007). Effects of beta-alanine supplementation on the onset of neuromuscular fatigue and ventilatory threshold in women. *Amino Acids* **32,** 381–386.

Swietach P, Youm J-B, Saegusa N, Leem C-H, Spitzer KW & Vaughan-Jones RD (2013). Coupled Ca2+/H+ transport by cytoplasmic buffers regulates local Ca2+ and H+ ion signaling. *Proc Natl Acad Sci U S A* **110,** E2064–73.

Tamaki N, Kaneko M, Mizota C, Kikugawa M & Fujimoto S (1990). Purification, characterization and inhibition of d-3-aminoisobutyrate aminotransferase from the rat liver. *Eur J Biochem* **189,** 39–45.

Tamaki N, Morioka S, Ikeda T, Harada M & Hama T (1980). Biosynthesis and degradation of carnosine and turnover rate of its constituent amino acids in rats. *J Nutr Sci Vitaminol* **26,** 127–139.

Teufel M, Saudek V, Ledig J-P, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C & Smirnova T (2003). Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* **278,** 6521–6531.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3,** RESEARCH0034.

Veiga-da-Cunha M, Chevalier N, Stroobant V, Vertommen D & Van Schaftingen E (2014). Metabolite proofreading in carnosine and homocarnosine synthesis: molecular identification of PM20D2 as β-alanyl-lysine dipeptidase. *J Biol Chem* **289,** 19726–19736.

Wallach D (1961). Studies on the GABA pathway. *Biochem Pharmacol* **5,** 323–331.

Study 2

Effects of histidine and beta-alanine supplementation on human muscle carnosine storage

Blancquaert L, Everaert I, Missinne M, Baguet A, Stegen S, Volkaert A, Petrovic M, Vervaet C, Achten E, De Maeyer M, De Henauw S, Derave W

Med Sci Sports Exerc (accepted for publication)

ABSTRACT

Purpose. Carnosine is a dipeptide composed of beta-alanine and L-histidine and is present in skeletal muscle. Chronic oral beta-alanine supplementation can induce muscle carnosine loading and is therefore seen as the rate-limiting factor for carnosine synthesis. However, the effect of L-histidine supplementation on carnosine levels in humans is never established. This study aims to investigate whether 1) L-histidine supplementation can induce muscle carnosine loading and 2) combined supplementation of both amino acids is more efficient than beta-alanine supplementation alone.

Methods. Fifteen male and 15 female participants were equally divided in three groups. Each group was supplemented with either pure beta-alanine (BA) (6g/day), L-histidine (HIS) (3.5g/day) or both amino acids (BA+HIS). Before (D0), after 12 (D12) and after 23 days (D23) of supplementation, carnosine content was evaluated in soleus and gastrocnemius medialis muscles by ^{1}H-MRS and venous blood samples were collected. Muscle biopsies were taken at D0 and D23 from the vastus lateralis. Plasma and muscle metabolites (beta-alanine, histidine, carnosine) were measured by HPLC.

Results. Both BA and BA+HIS groups showed increased carnosine concentrations in all investigated muscles, with no difference between these groups. In contrast, carnosine levels in the HIS group remained unaltered. Histidine levels were significantly decreased in plasma (-30.6%) and muscle (-31.6%) of the BA group, while this was prevented when beta-alanine and L-histidine were supplemented simultaneously.

Conclusion. We confirm that beta-alanine, and not L-histidine, is the rate-limiting precursor for carnosine synthesis in human skeletal muscle. Yet, although L-histidine is not rate-limiting, its availability is not unlimited and gradually declines upon chronic beta-alanine supplementation. The significance of this decline still needs to be determined, but may affect physiological processes such as protein synthesis.

KEYWORDS: Carnosine loading; ergogenic supplements; skeletal muscle metabolism

INTRODUCTION

In recent years, there has been increased research interest in the role of carnosine (beta-alanyl-L-histidine) in skeletal muscle metabolism and the supplementation with beta-alanine by athletic populations to increase concentrations of this muscle dipeptide (Blancquaert *et al.*, 2015). Carnosine is synthesized from the amino acids L-histidine and beta-alanine by carnosine synthase in several tissues, but displays its highest concentrations in skeletal muscles (5-8mmol/l wet weight in humans) (Boldyrev *et al.*, 2013). Harris and co-workers (2006) were the first to show that chronic oral ingestion of beta-alanine is able to elevate muscle carnosine content, supporting the assumption that beta-alanine is the rate-limiting precursor for carnosine synthesis in muscle. Since then, there have been consistent reports that chronic supplementation with beta-alanine (4-10 weeks, 4-6.4g/day) can increase muscle carnosine content by 40-100% (Hill *et al.*, 2007; Baguet *et al.*, 2009). Furthermore, raised muscle carnosine concentrations are associated with performance-enhancing (ergogenic) effects on high-intensity exercise, which is most likely attributable to carnosine's role as proton buffer (Baguet *et al.*, 2010*b*), calcium regulator (Dutka *et al.*, 2012) or a combination of these functions (Swietach *et al.*, 2013) in skeletal muscle.

Although beta-alanine has become a popular supplement as a result of these findings, direct comparison of the precursor availability of beta-alanine versus L-histidine in the synthesis of carnosine in human skeletal muscle is still unexplored. Stegen et al. (2013*a*) demonstrated that the incorporation efficiency of chronic orally ingested beta–alanine into muscle carnosine is very low (around 3%), suggesting that other determinants of carnosine synthesis may so far have been overlooked. Indeed, one explanation comes from our recent findings that ingested beta-alanine is likely prioritized towards other metabolic routes, such as transamination and energy delivery (Blancquaert *et al.*, 2016). Another possible factor is that the availability of L-histidine is more important than originally suggested by Harris et al. (2006). Their study found no significant difference in the increase in muscle carnosine content following a 4-week supplementation period with beta-alanine (total dose of 145.6g) or an isomolar dose of carnosine (total dose of 364g, corresponding to 143.3g beta-alanine), suggesting that the synthetic pathway is mainly driven by beta-alanine availability.

Similar conclusions came from an equine study (Dunnett & Harris, 1999) in which the authors observed that the concentration of muscle histidine (0.21mmol/l wet weight in equine muscle) is high relative to its Michaelis Constants (Km) for carnosine synthase (16.8µM) (Horinishi *et al.*, 1978), while the opposite is true for beta-alanine (0.15mmol/l wet weight concentration in equine muscle vs Km of 1-2.3mM) (Ng & Marshall, 1978; Kish *et al.*, 1979). However, Drozak et al. (2010) recently investigated the kinetic properties of recombinant human carnosine synthase and reported a Km which is much lower for beta-alanine (0.09mM) and higher for histidine (0.37mM) than the results previously calculated from partly purified enzyme extracts from the brain and central nervous system of mice (Horinishi *et al.*, 1978), rats (Ng & Marshall, 1978) or humans (Kish *et al.*, 1979). This would explain the accumulating number of papers which have attributed a more important role to dietary histidine in the carnosine homeostasis in different animal species. For example, a histidine-supplemented diet has been shown to increase the carnosine content of rats and broiler chickens by 35%-175% (Tamaki *et al.*, 1977, 1984; Haug *et al.*, 2008; Kralik *et al.*, 2015; Kai *et al.*, 2015). Furthermore, the carnosine and anserine (methylated analogue of carnosine) concentrations in broiler breast meat was enhanced in histidine-supplemented birds, while beta-alanine supplementation failed to have an effect alone or in addition to histidine (Park *et al.*, 2013). Together, these results implicate that histidine is the rate-limiting factor for carnosine synthesis in at least some animal species.

Although the carnosine metabolism differs between primates, birds and rodents, these data warrant further investigation in humans on the effect of chronic histidine supplementation on muscle carnosine loading. It is possible, even if histidine supplementation fails to directly increase muscle carnosine concentrations in humans, that chronic beta-alanine supplementation in humans may gradually deplete muscle histidine via a failure of dietary intake to match the increasing use of histidine (semi-essential, proteinogenic amino acid) for carnosine synthesis. If that is true, histidine availability could become rate-limiting for carnosine synthesis under conditions of chronic beta-alanine use and carnosine loading would be more efficient when chronic beta-alanine supplementation is combined with histidine. This theory has not been investigated to date. Accordingly, our aim was to study the effect of histidine

supplementation, alone or combined with beta-alanine, on human muscle carnosine content. We hypothesized that 1) histidine supplementation alone can induce muscle carnosine loading and 2) combined supplementation of both amino acids is more efficient toward muscle carnosine loading than beta-alanine supplementation alone. As underlying mechanism to the latter, we expect that chronic beta-alanine supplementation would gradually deplete histidine concentrations in plasma and muscle.

MATERIALS AND METHODS

Subjects

Thirty subjects (age: 20.0 ± 2.4 yr, body weight: 66.0 ± 10.6 kg, height: 172.8 ± 8.2 cm, BMI: 22.0 ± 2.7), both male (n = 15) and female (n = 15), participated in this study. All subjects were in good health and none of the participants were vegetarian. All subjects were non-specifically trained, but some of them took part in some form of recreational exercise 1–3 times per week. 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 and sample collection

The subjects were divided in three groups of 10 participants (with 5 men and 5 women in each group) and were matched for age, body weight, height and baseline carnosine concentrations in soleus and gastrocnemius (Table 1). Each group was supplemented with either pure beta-alanine (BA: 6g/day or 67.3mmol/day), L-histidine hydrochloride monohydrate (HIS: 4.7g/day which is equivalent to 3.5g/day L-histidine or 22.4mmol/day) or combined supplementation of both amino acids (BA+HIS: 67.3 + 22.4mmol/day). Because we hypothesized that histidine may become rate-limiting when beta-alanine is chronically ingested, we chose a 3:1 molar ratio of beta-alanine to histidine in the combined supplementation condition. Supplements were divided in 6 doses throughout the day (2x500mg capsules for BA, 2x390mg capsules for HIS) with at least 2h interval between intake occasions and participants were asked to take the supplements together with meals or snacks. The supplementation period lasted 23 days. To check for compliance, participants were asked to fill in a weekly diary, stating when they took their pills and consumed meals or snacks. Measurements were performed prior to the study (D0), after 12 days (D12) and at the end of the supplementation period (D23). At D0, D12 and D23, carnosine content was evaluated in soleus and gastrocnemius medialis muscles by 1H-MRS. Fasted venous blood samples (EDTA) were collected and immediately centrifuged to separate plasma, which was frozen at -20°C until subsequent analysis. Furthermore, muscle biopsies were taken at D0 and D23 from the left vastus lateralis at rest. Following local anaesthesia, a muscle sample was taken with a 12 Gauge true-cut

biopsy needle (Bard Magnum Biopsy gun; Bard, Inc., New Jersey, USA). The samples were then immediately frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

Table 1. Baseline values of the three experimental groups.

	BA	HIS	BA+HIS
Number of subjects	10	10	10
Age, yr	19.1 ± 1.3	20.5 ± 3.2	20.4 ± 2.4
Height, cm	171.4 ± 7.3	172.9 ± 9.6	174.1 ± 8.3
Weight, kg	63.1 ± 10.4	65.0 ± 11.3	69.9 ± 9.9
Carnosine soleus, mM	4.49 ± 1.23	4.30 ± 1.23	4.60 ± 1.04
Carnosine gastrocnemius, mM	7.22 ± 2.54	7.23 ± 1.59	7.32 ± 1.58

Values are mean ± SD

Food Questionnaire

Prior to the start of the supplementation period, subjects were asked to complete a 3-day food diary. To capture weekly variations, participants were asked to report 2 weekdays and 1 weekend day. Subjects needed to write down everything they consumed throughout the day and specify the quantities of each product. All diaries were checked for accuracy at least once and when information was missing (e.g. no quantities, no exact recipe…), this was requested during the subjects' first visit. Based on the food diaries, daily intake of calories, proteins, carbohydrates and histidine was determined by a professional dietician. As the Belgian food composition tables do not give any information on amino acid level, the amount of histidine intake was estimated based on the Danish, American (USDA) or German (BLS) food composition tables. For each of these, the ratio of histidine on protein was calculated for all food items. This information was used to estimate the daily histidine intake.

Determination of plasma and muscle metabolites by HPLC

Muscle biopsies of the vastus lateralis were dissolved in phosphate buffered saline solution (PBS, 30µL/1mg muscle tissue). Muscle homogenates and plasma of all participants were deproteinized using 35% sulfosalicylic acid (SSA) and centrifuged (5 min, 16,000 g). Deproteinized plasma supernatant (2.6µL) was mixed with 77.4µL of AccQ Fluor Borate buffer and 20µL of reconstituted Fluor Reagent from the AccQTag chemistry kit (Waters). For muscle homogenates, 5µL of deproteinized supernatant was mixed with

75µL AccQ Fluor Borate buffer. The same method was applied to the combined standard solutions of beta-alanine, carnosine, histidine and taurine (Sigma). The derivatized samples were applied to a Waters HPLC system with following parameters: XBridge BEH column (4.6 x 150 mm, 2.5µm) for carnosine, taurine and histidine quantification and AccQTag column (3.9 x 150 mm, 5 µm) for beta-alanine quantification, both with fluorescence detector (excitation/emission wavelength: 250/395 nm). The column was equilibrated with buffer A [10% eluent A (Waters) – 90% H2O], buffer B (100% acetonitrile) and buffer C (100% H2O) at a flow rate of 1ml/min at room temperature. BETA-alanine, histidine and taurine were measured in plasma, and together with these metabolites, carnosine was additionally measured in muscle.

Determination of muscle carnosine by ^{1}H-MRS

The carnosine content of all subjects was measured at D0, D12 and D23 by proton magnetic resonance spectroscopy (^{1}H-MRS) in soleus and gastrocnemius muscles, as previously described (Baguet et al., 2010). The subjects were lying in supine position 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 spherical knee-coil. Single voxel point-resolved spectroscopy (PRESS) sequence with the following parameters was used: repetition time (TR) = 2.000 ms, echo time (TE) = 30 ms, number of excitations = 128, 1.024 data points, spectral bandwidth of 1.200 Hz, and a total acquisition time of 4.24 min. The average voxel size was 40mm x 11 mm x 29 mm for soleus and 40 mm x 11 mm x 30 mm for gastrocnemius and the line width of the water signal was on average 25.3 Hz (soleus) and 28.0 Hz (gastrocnemius), following shimming procedures. The absolute carnosine content (in millimolar; mM) was calculated as described before by Baguet et al., 2010.

Confirmation of muscle histidine measurements following beta-alanine supplementation

In order to confirm and strengthen our results, histidine concentration was additionally measured on vastus lateralis muscle samples of a previous study performed in our lab (Stegen *et al.*, 2013*a*). In this study, subjects were supplemented with 3.2g beta-alanine/day (either pure or slow-release) during 6-7 weeks. Muscle histidine levels were measured by HPLC in 13 male subjects of this study.

Statistics

A one-way analysis of variance (ANOVA) was performed to compare the difference in baseline values (age, length, weight, muscle carnosine concentration) between the three groups. A 3 x 3 repeated measures analysis of variance (RM ANOVA) was used to evaluate muscle carnosine content by 1H-MRS and plasma metabolite concentrations with 'group' (BA; HIS; BA+HIS) as between-subjects factor and 'time' (D0; D12 and D23) as a within-subjects factor. When a significant interaction effect was present, analyses were repeated for each group separately and pairwise comparisons were used to compare the different time points. A 3 x 2 RM ANOVA was used to evaluate muscle biopsy metabolite concentrations with 'group' (BA; HIS; BA+HIS) as between-subjects factor and 'time' (D0; D23) as a within-subjects factor. When a significant interaction effect was present, pairwise comparisons were performed to compare D0 to D23 for each group separately. A paired T-test was used to evaluate the effect of beta-alanine supplementation on muscle histidine levels in the replication study. All statistical analyses were performed using SPSS 23.0 software (SPSS, Chicago, IL, USA). Values are presented as mean ± SD and significance was assumed at $p \leq 0.05$.

RESULTS

Muscle carnosine content

Both BA and BA+HIS groups showed significantly increased carnosine concentrations after 23 days of supplementation in all investigated muscles (BA: soleus $p < 0.001$ (+52.8%), gastrocnemius $p = 0.002$ (+29.0%), vastus lateralis $p = 0.010$ (+27.8%). BA+HIS: soleus $p < 0.001$ (+50.0%), gastrocnemius $p = 0.004$ (+31.5%), vastus lateralis $p = 0.043$ (+29.7%)) and there were no differences in the amount of loading between these two groups (Fig 1A-C).

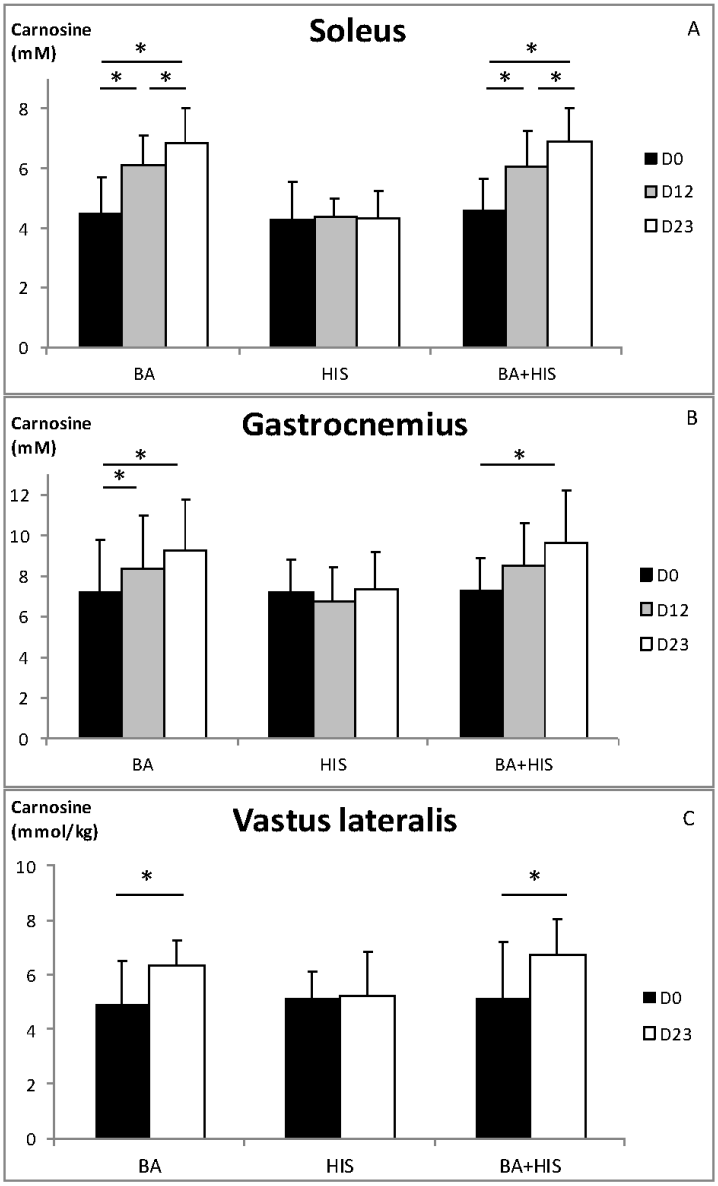

Figure 1. Effect of supplementation of BA, HIS or BA+HIS on carnosine concentrations in soleus (A), gastrocnemius (B) and vastus lateralis (C) muscles. * $p < 0.05$ for post-hoc analysis when a significant interaction effect was found

By taking into additional account the intermediate ^{1}H-MRS measurement (at D12, thus for soleus and gastrocnemius only), figure 2 displays muscle carnosine accretion in the first (D0-D12) versus the second half (D12-D23) of the supplementation period. Although there was no significant interaction effect, there was a 43% smaller carnosine accretion in the second half (D12-D23: 0.81 mmol/kg) compared to the first half (D0-D12: 1.42 mmol/kg) of the supplementation period in the BA group, but this decline in efficiency seemed less pronounced (-17%) in the BA+HIS group (ΔD12-D23: 1.11 mmol/kg vs ΔD0-D12: 1.34 mmol/kg) (Fig 2). In contrast to BA and BA+HIS groups, carnosine concentrations of the HIS group remained unaltered during the supplementation period (soleus p = 0.864 (+0.9%), gastrocnemius p = 0.771 (+2.2%), vastus lateralis p = 0.788 (+2.2%)) (Fig 1A-C).

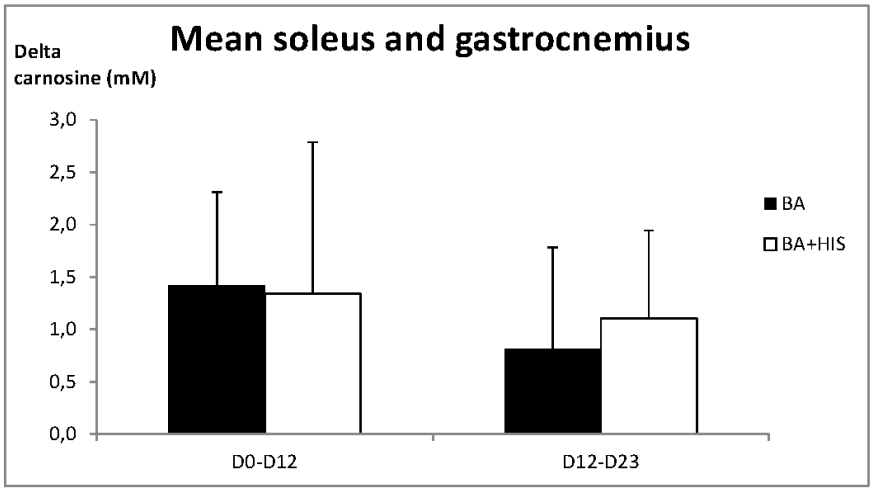

Figure 2. Change in carnosine levels in the first (D0-D12) and second (D12-D23) part of the supplementation period for BA and BA+HIS groups. Delta carnosine is calculated for the mean carnosine concentrations in soleus and gastrocnemius

Plasma and muscle histidine concentrations

The changes in both plasma and muscle histidine levels over time were dependent on the supplementation group (interaction effect p = 0.05 for plasma and p = 0.01 for muscle). Post-hoc statistical analysis revealed that in the BA group, histidine concentration was significantly decreased in plasma (55.0 ± 20.0 to 38.1 ± 15.0 µM at D23, p = 0.014, -30.6%) and muscle (0.20 ± 0.06 to 0.14 ± 0.09 mmol/kg at D23, p = 0.003, -31.6%). This decline was prevented when both BA and HIS were supplemented simultaneously (plasma: 52.1 ± 22.0 to 51.7 ± 18.2 µM, p = 0.949, -0.8%; muscle: 0.20 ± 0.12 to 0.21 ± 0.12 mmol/kg, p = 0.486, +6.2%). When histidine was supplemented alone, a slight, yet non-significant

increase in plasma (51.3 ± 14.4 to 62.7 ± 24.4 µM, p = 0.135, +22.2%) and muscle (0.19 ± 0.07 to 0.27 ± 0.12 mmol/kg, p = 0.153, + 38.8%) histidine levels was observed (Fig 3A&B; Table 2).

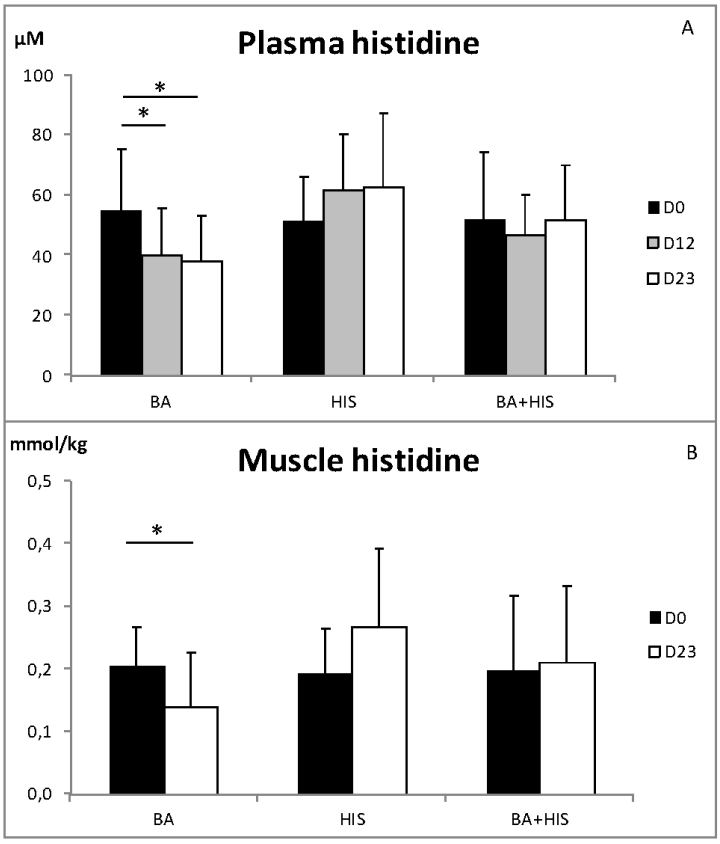

Figure 3. Effect of supplementation of BA, HIS or BA+HIS on histidine concentrations in plasma (A) and muscle (B). * p < 0.05 vs D0 for post-hoc analysis when a significant interaction effect was found

Plasma and muscle beta-alanine concentrations

A significant interaction effect was found for fasting plasma beta-alanine (p = 0.007), demonstrating significantly increased levels in both BA (2.6 ± 1.2 to 7.0 ± 3.6 µM, p = 0.009, + 172.1%) and BA+HIS (2.6 ± 1.0 to 6.2 ± 3.6 µM, p = 0.003, +141.4%) groups, but not in the HIS group (2.5 ± 0.9 to 2.7 ± 1.1 µM, p = 0.385) after 23 days of supplementation (Table 2). The increase in the BA and BA+HIS groups was already established after 12 days of supplementation (p = 0.01 and p = 0.001 for both BA and BA+HIS, respectively), with no further increase in the second part of the supplementation period (p = 0.237 and p = 0.370 for BA and BA+HIS, respectively). In contrast, no significant interaction effect was found for muscle beta-alanine, yet both BA and BA+HIS groups showed a slight, non-significant increase in muscle beta-alanine levels (BA: 0.12 ±

0.08 to 0.19 ± 0.06 mmol/kg; BA+HIS 0.14 ± 0.14 to 0.21 ± 0.12 mmol/kg) while this remained stable in the HIS group (0.11 ± 0.06 to 0.12 ± 0.09 mmol/kg) (Table 2).

Plasma and muscle taurine concentrations

No significant interaction effect was found for plasma or muscle taurine levels ($p = 0.063$ and $p = 0.156$, respectively). Yet, both the BA and BA+HIS groups seemed to undergo a slight, non-significant decrease in plasma taurine levels (BA: 52.7 ± 7.8 to 39.3 ± 10.0 µM at D23, -25.3%; BA+HIS: 53.1 ± 17.9 to 42.4 ± 12.2 µM at D23, -20.1%), which was not the case for the HIS group (45.7 ± 16.9 to 51.2 ± 12.8 µM at D23, +12.1%). In muscle, a slight, non-significant decrease in taurine levels was only observed in the BA group (11.3 ± 2.6 to 9.8 ± 1.5 mmol/kg at D23, -13.5%), while muscle taurine remained stable in both the HIS and BA+HIS groups (HIS: 11.5 ± 2.5 to 12.3 ± 5 mmol/kg, +7.4%; BA+HIS: 10.0 ± 2.0 to 10.0 ± 2.0 mmol/kg, -0.2%) (Table 2).

Confirmation of beta-alanine induced muscle histidine decline

In order to replicate and confirm the finding of the beta-alanine-induced decline in muscle histidine concentration, we measured the histidine concentration on muscle samples of 13 subjects of a previous study performed in our lab (Stegen *et al.*, 2013*a*). Muscle histidine concentration decreased from 0.36 ± 0.10 to 0.28 ± 0.07 mmol/kg ($p = 0.02$, -23.2%), confirming the decline in muscle histidine after beta-alanine supplementation found in the main study.

Table 2. Plasma and muscle beta-alanine, histidine and taurine concentrations for the three experimental groups at D0, D12 and D23.

Group		**BA**			**HIS**			**BA+HIS**		
	Timepoint	*D0*	*D12*	*D23*	*D0*	*D12*	*D23*	*D0*	*D12*	*D23*
beta-alanine	plasma (µM)	2.6 ± 1.2	5.8 ± 2.4*	7.0 ± 3.6*	2.5 ± 0.9	2.5 ± 0.8	2.7 ± 1.1	2.6 ± 1.0	5.8 ± 3.0*	6.2 ± 3.6*
	M. vastus lateralis (mmol/kg)	0.12 ± 0.08	/	0.19 ± 0.06	0.11 ± 0.06	/	0.12 ± 0.09	0.14 ± 0.14	/	0.21 ± 0.12
Histidine	plasma (µM)	55.0 ± 20.0	40.1 ± 15.1*	38.1 ± 15.0*	51.3 ± 14.4	61.6 ± 18.5	62.7 ± 24.4	52.1 ± 22.0	46.8 ± 13.2	51.7 ± 18.2
	M. vastus lateralis (mmol/kg)	0.20 ±0.06	/	0.14 ± 0.09*	0.19 ± 0.07	/	0.27 ± 0.12	0.20 ± 0.12	/	0.21 ± 0.12
Taurine	plasma (µM)	52.7 ± 7.8	46.6 ± 12.5	39.3 ± 10.0	45.7 ± 16.9	49.2 ± 12.6	51.2 ± 12.8	53.1 ± 17.9	45.2 ± 15.1	42.4 ± 12.3
	M. vastus lateralis (mmol/kg)	11.3 ± 2.6	/	9.8 ± 1.5	11.5 ± 2.5	/	12.3 ± 5.0	10.0 ± 2.0	/	10.0 ± 2.0

Muscle values (mmol/kg wet weight) were obtained from muscle biopsies, which were taken at D0 and D23. Values are mean ± SD. * $p < 0.05$ vs D0 for post-hoc analysis in case a significant interaction effect was found

Dietary registration

There was no significant difference between the 3 experimental groups concerning the daily intake of calories, proteins, carbohydrates and histidine through the regular diet. The mean intake of histidine averaged 2.66 ± 0.90g/d, which corresponds to 0.040 ± 0.011 g HIS/kg BW/d. Supplementing 3.5g histidine per day therefore resulted in a daily intake which was 2.5 times higher compared to the food-based dietary intake (Table 3).

Table 3. Mean daily self-reported nutritional intake of calories, proteins and histidine of the three experimental groups.

	Calories (kcal/d)	Protein (g/kg BW/d)	Histidine (g/kg BW/d)	Histidine (g/d)	Histidine supplement (g/d)	Total histidine (g/d)
BA	2536 ± 886	1.47 ± 0.45	0.041 ± 0.01	2.6 ± 1.1	0	2.6
HIS	2354 ± 449	1.49 ± 0.31	0.042 ± 0.01	2.7 ± 0.7	3.5	6.2
BA+HIS	2471 ± 552	1.36 ± 0.39	0.038 ± 0.01	2.6 ± 0.9	3.5	6.1

Values were calculated based on the 3-day food diary. Values are mean ± SD. SD refers to total daily variability between the different subjects per group

DISCUSSION

To the best of our knowledge, this is the first study to investigate the effect of chronic oral histidine supplementation on muscle carnosine content in humans. Unlike the findings in some animals, histidine supplementation did not induce carnosine loading in human muscles, although it tended to increase muscle histidine content, suggesting that elevated tissue histidine in itself is not sufficient to raise carnosine. In different animal species, it was already repeatedly demonstrated that animals fed a histidine-deficient diet show a reduction of muscle carnosine content of 60 up to 90% (Quinn & Fisher, 1977; Tamaki *et al.*, 1977, 1984; Amend *et al.*, 1979) and that histidine supplementation alone can increase carnosine levels at least in some species (Tamaki *et al.*, 1977, 1984; Haug *et al.*, 2008; Park *et al.*, 2013; Kralik *et al.*, 2015; Kai *et al.*, 2015). The discrepancy between human vs bird and rodent data suggest that carnosine metabolism differs between these species when it comes to the rate-limiting precursor for carnosine synthesis.

In order to investigate whether histidine availability remains sufficient despite continued supplementation of only one of the precursors (beta-alanine) of carnosine synthesis, we monitored histidine levels during a period of beta-alanine supplementation. Interestingly, the current experiment together with the re-analysis of muscle samples from a previous study of our laboratory (Stegen *et al.*, 2013*a*) elucidated that both muscle and plasma histidine levels showed a substantial decline (≈-30%) with beta-alanine supplementation. This indicates that histidine availability is, although initially not rate-limiting, certainly not unlimited in humans either. However, this does not seem problematic for carnosine synthesis efficiency in the first 12 days of supplementation, as co-supplementation with histidine prevents the histidine decrease without affecting the amount of carnosine loading. Yet, although no significant interaction effect was found, there seemed to be a more modest decline in the amount of carnosine accretion during the second part of supplementation (D12-D23) in the BA+HIS group compared to the BA group. As the supplementation period in this study only lasted 23 days with a total ingested beta-alanine dose of 138g, one could speculate that the depletion of plasma and muscle histidine would become more problematic in longer duration studies with higher amounts of supplemented beta-alanine. In practice, guidelines for beta-alanine mostly recommend 4 to 6 or even up to 10 weeks of supplementation (Stellingwerff *et al.*, 2012*b*), containing

total ingested beta-alanine doses ranging from 179.2 up to 414.4g (Hill *et al.*, 2007; Kendrick *et al.*, 2008; Baguet *et al.*, 2009; Chung *et al.*, 2012, 2014; Danaher *et al.*, 2014). The study of Hill et al. (2007) measured carnosine loading in the vastus lateralis after 4 and 10 weeks of beta-alanine supplementation, corresponding to a total ingested beta-alanine dose of 145.6g and 414.4g, respectively. They reported an increase in carnosine levels of 58.8% at 4 weeks and 80.1% at 10 weeks, demonstrating a lower loading efficiency in the second period of supplementation. It remains to be established whether the attenuated carnosine loading upon continued beta-alanine supplementation for several months could possibly be related to gradually further depleted histidine levels and thus whether histidine supplementation can be of benefit during more prolonged beta-alanine supplementation.

In strength-training athletes in an anabolic state, depleted histidine stores as a result of beta-alanine supplementation could become problematic. Several studies have explored the ergogenic potential of beta-alanine supplementation as a training aid during strength training (Kendrick *et al.*, 2008; Bellinger, 2014). The rationale holds that elevated muscle carnosine content would better maintain myocellular homeostasis (e.g. in pH) during resistance exercise, and thereby increase the number of contraction repetitions (training volume) and subjective feeling of fatigue, as suggested by Hoffman et al. (2008). However, Kendrick et al. (2008) were not able to demonstrate an added benefit of chronic beta-alanine supplementation during a 10-week resistance training program on muscle strength or muscle mass gains. Possibly, an unnoticed decline in muscle histidine concentration may have partly antagonized the potential added benefit of elevated muscle carnosine during strength training. As such, a decrease in histidine levels could negatively affect the stimulation of muscle protein synthesis which is fundamentally regulated by extracellular and intracellular amino acid availability (Kimball & Jefferson, 2002). Habitual protein intakes of strength athletes is on average 2g/kg BW/day (Phillips, 2004), corresponding with approximately 3.7g of L-histidine per day for a 66 kg person (mean weight of subjects in this study), which is more similar to the daily intake of the non-training subjects of the BA group of this study (2.61g/d), compared to the intake of the BA+HIS group (6.14g/d). Therefore, given their anabolic state, the higher dietary protein intake in strength-training athletes may possibly not compensate for the

reduction in muscle histidine when combining strength training with beta-alanine supplementation.

Another example in which the human body is in an anabolic state is during growth. Due to the demands of growth and development, getting adequate protein is important during infancy, childhood, and adolescence (Joint WHO/FAO/UNU Expert Consultation., 2007). Some inconsistency exists regarding the categorization of L-histidine, but it is mostly categorized as a semi-essential amino acid (Stifel & Herman, 1972) because adults generally produce adequate amounts but children may not. Given this, the use of beta-alanine supplementation in children, especially during puberty, should be strongly discouraged.

Likewise, adults suffering from obesity and metabolic syndrome seem to have an increased need for dietary histidine. In a well-powered intervention study, Feng et al. (2013) demonstrated marked reductions in insulin resistance and inflammatory markers over a 12-week period of 4g/day of oral L-histidine supplementation in obese women. Therefore, supplementing beta-alanine in a condition of insulin resistance or obesity may be inappropriate as this may further decline histidine stores, which are already compromised in these pathologies, as suggested by Feng et al. (2013). Indeed, a recent study indicated beneficial effects of oral carnosine but not of beta-alanine supplementation on markers of metabolic stress in high-fat fed rats, despite both strategies increased muscle carnosine content to the same extent (Stegen *et al.*, 2015). Similar positive effects of oral carnosine supplementation, which in fact equalizes combined beta-alanine and L-histidine supplementation in a 1:1 molar ratio, have recently also been suggested in obese humans (DeCourten *et al.*, 2016). Thus, even though chronic oral beta-alanine supplementation may have applications beyond the sports arena, such as in the elderly population (DelFavero *et al.*, 2012), the current study suggests that co-supplementing with histidine could be a better alternative in some instances.

A possible exercise-related metabolic pathway in which histidine is involved, is histamine synthesis through decarboxylation of L-histidine by histidine decarboxylase (HDC). Some beneficial exercise-related roles are ascribed to histamine such as the vasodilating effect

on arterioles in (post-)exercise hyperaemia (Jones, 2016). Furthermore, Romero et al. (2016) demonstrated that the human response to exercise includes an altered expression of thousands of protein-coding genes, and >25% of this response is driven by histamine. In general, it is believed that histamine can function in a paracrine or endocrine fashion or can be stored in mast cells. Mice deficient in HDC or histamine H1-receptors displayed markedly reduced endurance abilities in comparison to control mice and the anti-fatigue effect of endogenous histamine is completely intact in mast-cell-deficient mice (Endo *et al.*, 1998; Niijima-Yaoita *et al.*, 2012), suggesting that histamine might also derive from muscle histidine. HDC is expressed in human skeletal muscle and is upregulated following exercise (Romero *et al.*, 2016). Decreased muscle histidine levels following beta-alanine supplementation might therefore also affect histamine production and thus exercise responses. Overall, it remains to be investigated to what extent depleted muscle and plasma histidine levels following beta-alanine supplementation affect carnosine metabolism, protein and insulin metabolism or histidine decarboxylase kinetics.

A limitation of this study is the fact that only one histidine dose was tested, alone or in combination with beta-alanine. This implicates that the conclusions cannot directly be generalized to higher histidine doses or other beta-alanine:histidine ratios than the 3:1 ratio tested in this study. We hypothesized that histidine may become rate-limiting with chronic and intensive beta-alanine consumption, suggesting a smaller amount of histidine compared to beta-alanine should suffice to support the beta-alanine intake. Therefore, we chose a histidine dose in the same range than the dose applied in a previous intervention study and for which no side-effects were reported (Feng *et al.*, 2013). Still, the dose used in this study (3.5g/d) was required to considerably increase the daily intake of histidine through the regular diet (2.66g/d on average in this study, which was thus at least doubled by the supplemented histidine dose). The use of 3-day food diaries to estimate histidine intake is a second limitation. It is generally believed that precise estimates of protein and energy can be done with 3-day food diaries (Basiotis *et al.*, 1987), but amino acids probably show higher day-to-day variability, suggesting that the level of precision is lower for this parameter. Yet, the dietary histidine levels were measured primarily to 1) provide context for the supplemented versus normal dietary dose of histidine ingestions, and 2) control at group level whether the intake differs between the

intervention groups, which is probably only minimally influenced by a lower precision. Moreover, it should be acknowledged that the absorption kinetics may differ between pure and food-derived histidine intake, but no literature is currently available on this topic.

In summary, we have shown that histidine supplementation alone did not suffice to increase muscle carnosine content and adding histidine to the beta-alanine supplementation protocol did not improve short-term loading efficiency, confirming the rate-limiting role of beta-alanine. However, we equally demonstrated that chronic beta-alanine supplementation reduces plasma and muscle histidine levels, which could be prevented by co-supplementing L-histidine alongside beta-alanine. It remains to be determined whether the depletion of histidine levels by beta-alanine can compromise physiological processes such as carnosine loading of longer duration or protein synthesis in an anabolic state.

ACKNOWLEDGEMENTS

The paper is dedicated to Dr. Birgit Wuyts, who was instrumental in the design of the experiments, but sadly passed away during the course of the study. The stimulating discussion of the results with Dr. Roger Harris is greatly appreciated. This study was financially supported by grants from the Research Foundation – Flanders (FWO G.0243.11 and G.0352.13N) to WD. LB is a recipient of a PhD Scholarship and IE is a recipient of a post-doctoral scholarship by the Research Foundation – Flanders (FWO). The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

REFERENCES

Amend J, Strumeyer D, Fisher H. Effect of dietary histidine on tissue concentrations of histidine-containing dipeptides in adult cockerels. *J Nutr* 1979;109:1779–86.

Baguet A, Koppo K, Pottier A, Derave W. Beta-alanine supplementation reduces acidosis but not oxygen uptake response during high-intensity cycling exercise. *Eur J Appl Physiol* 2010;108(3):495–503.

Baguet A, Reyngoudt H, Pottier A, et al. Carnosine loading and washout in human skeletal muscles. *J Appl Physiol* 2009;106(3):837–42.

Basiotis PP, Welsh SO, Cronin FJ, Kelsay JL, Mertz W. Number of days of food intake records required to estimate individual and group nutrient intakes with defined confidence. *J Nutr* 1987;117(9):1638–41.

Bellinger PM. β-Alanine Supplementation for Athletic Performance. *J Strength Cond Res* 2014;28(6):1751–70.

Blancquaert L, Baba S, Kwiatkowski S, et al. Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta-alanine transamination. *J Physiol* 2016;594(17):4849–63.

Blancquaert L, Everaert I, Derave W. Beta-alanine supplementation, muscle carnosine and exercise performance. *Curr Opin Clin Nutr Metab Care* 2015;18(1):63–70.

Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev* 2013;93(4):1803–45.

Chung W, Shaw G, Anderson ME, et al. Effect of 10 week beta-alanine supplementation on competition and training performance in elite swimmers. *Nutrients* 2012;4(10):1441–53.

Danaher J, Gerber T, Wellard RM, Stathis CG. The effect of β-alanine and NaHCO3 co-ingestion on buffering capacity and exercise performance with high-intensity exercise in healthy males. *Eur J Appl Physiol* 2014;114(8):1715–24.

DeCourten B, Jakubova M, De Courten MPJ, et al. Effects of carnosine supplementation on glucose metabolism: Pilot clinical trial. *Obesity* 2016;24(5):1027–34.

DelFavero S, Roschel H, Solis MY, et al. Beta-alanine (Carnosyn^{TM}) supplementation in elderly subjects (60-80 years): effects on muscle carnosine content and physical capacity. *Amino Acids* 2012;43(1):49–56.

Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V, Van Schaftingen E. Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem* 2010;285(13):9346–56.

Dunnett M, Harris RC. Influence of oral -alanine and L-histidine supplementation on the carnosine content of the gluteus medies. *Equine Vet J* 1999;30:499–504.

Dutka TL, Lamboley CR, McKenna MJ, Murphy RM, Lamb GD. Effects of carnosine on contractile apparatus Ca^{2+} sensitivity and sarcoplasmic reticulum Ca^{2+} release in human skeletal muscle fibers. *J Appl Physiol* 2012;112(5):728–36.

Endo Y, Tabata T, Kuroda H, Tadano T, Matsushima K, Watanabe M. Induction of histidine decarboxylase in skeletal muscle in mice by electrical stimulation, prolonged walking and interleukin-1. *J Physiol* 1998;509(2):587–98.

Feng RN, Niu YC, Sun XW, et al. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: A randomised controlled trial. *Diabetologia* 2013;56(5):985–94.

Harris RC, Tallon MJ, Dunnett M, et al. The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino Acids* 2006;30(3):279–89.

Haug A, Rodbotten R, Mydland LT, Christophersen OA. Increased broiler muscle carnosine and anserine following histidine supplementation of commercial broiler feed concentrate. *Acta Agric Scand Sect A-Animal Sci* 2008;58(2):71–7.

Hill CA, Harris RC, Kim HJ, et al. Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino Acids* 2007;32(2):225–33.

Hoffman JR, Ratamess NA, Faigenbaum AD, et al. Short-duration β-alanine supplementation increases training volume and reduces subjective feelings of fatigue in college football players. *Nutr Res* 2008;28(1):31–5.

Horinishi H, Grillo M, Margolis F. Purification and characterization of carnosine synthetase from mouse olfactory bulbs. *J Neurochem* 1978;31:909–19.

Joint WHO/FAO/UNU Expert Consultation. Protein and amino acid requirements in human nutrition. *WHO Tech Rep Ser* 2007;1–265.

Jones AW. Perspectives in Drug Development and Clinical Pharmacology: The Discovery of Histamine H 1 and H 2 Antagonists. *Clin Pharmacol Drug Dev* 2016;5(1):5–12.

Kai S, Watanabe G, Kubota M, Kadowaki M, Fujimura S. Effect of dietary histidine on contents of carnosine and anserine in muscles of broilers. *Anim Sci J* 2015;86(5):541–6.

Kendrick IP, Harris RC, Kim HJ, et al. The effects of 10 weeks of resistance training combined with beta-alanine supplementation on whole body strength, force production, muscular endurance and body composition. *Amino Acids* 2008;34(4):547–54.

Kimball SR, Jefferson LS. Control of protein synthesis by amino acid availability. *Curr Opin Clin Nutr Metab Care* 2002;5(1):63–7.

Kish S, Perry T, Hansen S. Regional distribition of homocarnosine, homocarnosine-

carnosine synthetase and homocarnosinase in human brain. *J Neurochem* 1979;32:1629–36.

Kralik G, Sak-Bosnar M, Kralik Z, Galovic O, Grcevic M, Kralik I. Effect of β-alanine and L-histidine on concentration of carnosine in muscle tissue and oxidative stability of chicken meat. *Poljoprivreda/Agriculture* 2015;21(1 Supplement):190–4.

Ng R, Marshall F. Regional and subcellular distribution of homocarnosine-carnosine synthetase in the central nervous system of rats. *J Neurochem* 1978;30:187–90.

Niijima-Yaoita F, Tsuchiya M, Ohtsu H, et al. Roles of histamine in exercise-induced fatigue: favouring endurance and protecting against exhaustion. *Biol Pharm Bull* 2012;35(1):91–7.

Park S, Kim C, Namgung N. Effects of dietary supplementation of histidine, β-alanine, magnesium oxide, and blood meal on carnosine and anserine concentrations of broiler breast meat. *J Poult Sci* 2013;50:251–6.

Phillips SM. Protein requirements and supplementation in strength sports. *Nutrition* 2004;20(7-8):689–95.

Romero SA, Hocker AD, Mangum JE, et al. Evidence of a broad histamine footprint on the human exercise transcriptome. *J Physiol* 2016;(541):In press.

Stegen S, Blancquaert L, Everaert I, et al. Meal and beta-alanine coingestion enhances muscle carnosine loading. *Med Sci Sports Exerc* 2013;45(8):1478–85.

Stegen S, Stegen B, Aldini G, et al. Plasma carnosine, but not muscle carnosine, attenuates high-fat diet-induced metabolic stress. *Appl Physiol Nutr Metab* 2015;40(9):868–76.

Stellingwerff T, Decombaz J, Harris RC, Boesch C. Optimizing human in vivo dosing and delivery of β-alanine supplements for muscle carnosine synthesis. *Amino Acids* 2012;43(1):57–65.

Stifel F, Herman R. Is histidine an essential amino acid in man? *Am J Clin Nutr* 1972;25:182–5.

Swietach P, Youm J-B, Saegusa N, Leem C-H, Spitzer KW, Vaughan-Jones RD. Coupled Ca2+/H+ transport by cytoplasmic buffers regulates local Ca2+ and H+ ion signaling. *Proc Natl Acad Sci U S A* 2013;110(22):E2064–73.

Tamaki N, Funatsuka A, Wakabayashi M, Hama T. Effect of histidine-free and-excess diets on anserine and carnosine contents in rat gastrocnemius muscle. *J Nutr Sci Vitaminol (Tokyo)* 1977;23:331–40.

Study 3

Body creatine, but not carnitine and carnosine stores, decline by a 6-month vegetarian diet in omnivorous women

Blancquaert L, Baguet A, Bex T, Volkaert A, Everaert I, Delanghe J, Petrovic M, Vervaet C, Dehenauw S, Constantin-Teodosio Dumitru, Greenhaff P, Derave W

Am J Clin Nutr (in preparation)

ABSTRACT

Background: Balanced vegetarian diets are very popular and contain health-promoting characteristics, although they are nearly absent in creatine and carnosine and contain considerably less carnitine. Very few longitudinal intervention studies investigating the effect of a vegetarian diet on the metabolism of these compounds currently exist.

Objective: We aimed to investigate the effect of transiently switching omnivores onto a vegetarian diet for 6 months on the muscle and plasma creatine, carnitine and carnosine homeostasis.

Design: In a 6-month intervention, 40 omnivorous women were divided in three groups: control (continued omnivorous diet; Control), a vegetarian diet without supplementation (Veg+Pla), and a vegetarian diet combined with daily beta-alanine (0.8-0.4 g/day) and creatine supplementation (1 g creatine monohydrate/day) (Veg+Suppl). Before (0M) and after 3 (3M) and 6 months (6M), subjects performed an incremental cycling test and fasted venous blood samples, muscle biopsies and 24 hr urine samples were collected. Muscle carnosine content was determined by ^{1}H-MRS.

Results: Plasma creatine and muscle total creatine concentrations declined from 0M to 3M in the Veg+Pla group ($p = 0.013$ and $p = 0.025$, respectively), while both increased from 0M in the Veg+Suppl group ($p = 0.004$ and $p = 0.007$, respectively). None of the carnitine-related compounds in plasma or muscle showed a significant interaction effect. Muscle carnosine was unchanged over 6M in Control and Veg+Pla groups, but increased in the Veg+Suppl group in soleus ($p < 0.001$) and gastrocnemius ($p = 0.001$) muscle. VO_{2max} and time to exhaustion of the incremental cycling test did not differ between the experimental groups at baseline, neither did it change during the 6-month intervention period.

Conclusions: Body creatine concentrations declined over a 6-month vegetarian diet in omnivorous women, which was absent when accompanied by daily creatine supplementation, whereas carnitine and carnosine homeostasis is not affected by a 6-month vegetarian diet.

KEYWORDS: carninutrients, lacto-ovo-vegetarians, beta-alanine

INTRODUCTION

Vegetarian dietary patterns are becoming increasingly popular for various ecological, ethical or health-related reasons. A high consumption of red and processed meat has been consistently demonstrated to elevate mortality risk from certain cancers (1) and cardiovascular diseases (2,3). Yet, it is unclear whether the reduced mortality risk in lacto-ovo vegetarians, found in some studies, is related to the omission of meat and fish, or rather to more prevalent diet-unrelated health-conscious behaviour. A balanced lacto-ovo-vegetarian diet differs in nutrient intake from an omnivorous diet, e.g. by increased intake of fibre, magnesium and antioxidants, but lower intake of omega-3 fatty acids and vitamin B12 (4); all of which is reasonably well-documented in the scientific literature. However, the impact of the near absent intake of creatine, carnitine and carnosine in a vegetarian diet is less well established and could be relevant in relation to muscle function, exercise capacity and sports performance.

Creatine, carnitine and carnosine are solely or almost solely found in tissues of animals (5), with highest concentrations in skeletal muscle tissue (hence the name, derived from latin *carnis*, flesh). McCarty (5) therefore suggested to designate these compounds as “carninutrients”. They neither qualify as micronutrient, because they are ingested in relatively high amounts (range of 0.1-5 g per day), nor as macronutrient, because they are not primarily serving an energy provision role. Yet, creatine, carnitine and carnosine are nitrogenous molecules that possess properties with relevance to muscle function. Creatine plays a crucial bioenergetic role in tissues with high metabolic demand by rephosphorylating adenosine diphosphate (ADP) to synthesize adenosine triphosphate (ATP) (6). Carnitine is known to play a key role within several cellular energy producing pathways such as the transport of long-chain fatty acids into the mitochondria (7–9). It is of highest abundance in meat, however, it is also present in a number of non-meat nutrients (e.g. nuts, potatoes, milk). In contrast, the dipeptide carnosine (beta-alanyl-L-histidine) is exclusively present in meat and fish. Carnosine has a wide spectrum of bioactive properties such as pH-buffering, calcium regulation, antiglycation and antioxidant activity (10). Numerous studies in literature demonstrated the beneficial effects of creatine, carnitine (combined with carbohydrates) or beta-alanine (precursor amino-acid of carnosine) supplementation on exercise performance in athletes (11–13),

and therapeutic potential toward certain disorders in which muscle function is a key component (6,14,15).

Cross-sectional studies suggested that the homeostasis of these carninutrients in humans is negatively affected by long-term vegetarianism. Somewhat lower plasma carnitine concentrations in vegetarians than in omnivores are reported (16–19), but no consistency exists regarding muscle carnitine content. Stephens et al. (19) reported 17% lower muscle total carnitine concentrations in vegetarian women while no differences were found in same measure in the study of Novakova et al. (16). Until now, no human longitudinal intervention study is available investigating the effect of transiently switching omnivores onto a vegetarian diet on carnitine homeostasis. It is known, however, that carnitine has a slow turnover rate (300-500µmol/day) (7) and since it is still present in a vegetarian diet, it can be suggested that carnitine homeostasis is maintained during the first months of vegetarianism. Chronic and complete restriction of dietary creatine and carnosine is demonstrated to result in lower plasma creatine (17), and decreased intramuscular creatine (20) and carnosine (21,22) concentrations as compared with omnivorous subjects. Thus, these cross-sectional data suggest that the endogenous synthesis capacity may not be sufficient to maintain muscle creatine and carnosine concentrations, and therefore the latter may partly depend on dietary intake. Creatine is known to have a high turnover rate (~2g/day) (23). Consequently, a 3-week vegetarian intervention in omnivorous subjects already decreased muscle creatine content by 10% (24) and longer-term interventions might thus even deplete creatine to a greater extent. For carnosine, turnover rate is much slower compared to creatine (25). Accordingly, a 5-week vegetarian intervention study indicated no significant reduction in muscle carnosine content (26). However, in contrast to carnitine, carnosine is completely absent in vegetarian diets, suggesting that carnosine homeostasis might be affected by longer-term interventions.

This study aimed therefore to investigate the effect of a 6-month vegetarian diet on body creatine, carnitine and carnosine stores in omnivorous women. We hypothesized that homeostasis of creatine and carnosine would be disrupted when their dietary intake was lacking. For carnitine, however, we hypothesized that homeostasis can be maintained given its slow turnover rate and its presence in some non-meat nutrients. A second aim was to investigate whether supplementation of creatine and beta-alanine (the rate-

limiting precursor of carnosine synthesis), concurrently with a lacto-ovo-vegetarian diet, was able to correct for potentially emerging deficiencies.

MATERIALS AND METHODS

Subjects

Forty healthy female omnivores volunteered to participate in this 6-month intervention study. Exclusion criteria were smoking, chronic use of medication, athletes participating in competitions, vegetarianism or eating meat or fish less than 5 times a week. One woman dropped out within 2 months for personal reasons. At the start of the study, the subjects' age, weight, height and body mass index were 25.6 ± 7.3 yrs, 62.7 ± 7.9 kg, 167 ± 6 cm and 22.3 ± 2.6 kg/m^2, respectively. None of them were taking supplements containing creatine, carnosine, beta-alanine or carnitine in the 3 months prior to the start of the study. All participants gave their informed consent and the study was approved by the local ethics committee (Ghent University Hospital, Belgium).

Study design

The study was scheduled over a period of 6 months and measurements were performed 1 week prior to the intervention (0M), after 3 months (3M) and within the last week (6M). Ten women continued their omnivorous diet throughout the entire study (controls) and the other 29 subjects switched to a lacto-ovo-vegetarian diet for 6 months. The vegetarian group was split in 2 groups, matched for age, weight, height and baseline carnosine concentrations in soleus and gastrocnemius medialis muscles. Fourteen of them were supplemented with beta-alanine and creatine (Veg+Suppl) and the other 15 women received a placebo (Veg+Pla). With regard to supplementation, the study was double-blind placebo-controlled. The lacto-ovo-vegetarian diet consisted of vegetables, fruits, seeds, grains, meat substitutes, eggs and dairy products and the exclusion of meat, poultry and fish. Subjects were asked to complete a 3 day food diary at the start and after 3 months and received nutritional advice by a dietician during the study to prevent deficiencies in macronutrients and micronutrients. Furthermore, vegetarian recipes were provided by email to support the subjects in their vegetarian diet.

Supplements

The supplementation protocol included simultaneously daily oral administration of creatine monohydrate (Creapure®, AlzChem AG, Germany) and slow-release beta-alanine (Carnosyn®, Natural Alternatives International, San Marcos, USA) or a placebo

(maltodextrin, Natural spices, France). The Veg+Suppl group ingested 1 g of creatine monohydrate (2 capsules of 500 mg) and 0.8 g of beta-alanine (1 Carnosyn® tablet) each day for the first 3 months. Intermediate analysis of muscle carnosine content at 3 months revealed that 0.8 g of beta-alanine was too high to maintain carnosine at baseline concentration, and therefore the beta-alanine dose was reduced to 0.4 g/day during the last 3 months of the study, with creatine dosing remaining at 1 g/day. The Veg+Pla group was supplemented with an identical number of capsules and tablets of maltodextrin. All subjects were asked not to take any other supplements than those provided by the current study. Compliance was checked by asking the subjects to return the containers and counting the pills that were left. The control group, who remained on an omnivorous diet, did not receive any supplements.

Incremental cycling test

At 0, 3 and 6M, subjects performed an incremental cycling test. On the first visit, subjects were screened to be medically fit before starting the incremental cycling protocol to exhaustion. The test was performed on an electrically-braked cycling ergometer (Excalibur; Lode, Groningen, The Netherlands). Oxygen consumption was measured continuously via a computerized breath-by-breath system (JaegerOxycon Pro, Hoechberg, Germany). Following a 3 min warm-up at 40 W, the workload was increased by 40 W every 3 min until the point the subjects failed to continue to pedal at 60 rpm. Capillary blood samples were taken before and immediately after the incremental cycling test and pH and lactate was measured (blood gas analysis: GEM, Premier TM 3000, Instrumentation Laboratory, MA, USA).

Sample collection

Fasted venous blood samples were collected into heparin plasma tubes and serum gel tubes after 0, 3 and 6M. Heparin tubes were immediately centrifuged after collection to separate plasma. Serum tubes were left at rest at room temperature to obtain complete coagulation, before performing the same centrifugation process as for plasma. All samples were frozen at -20°C until subsequently analysis. True cut muscle biopsies were taken at baseline and after 3M from the vastus lateralis of the left leg at rest. Following local anesthesia of the skin and subcutaneous tissues with 5 mL lidocaine, two muscle samples were taken with a 14 Gauge true-cut biopsy needle (Bard Magnum Biopsy gun;

Bard, Inc., New Jersey, USA). The muscle samples were then immediately frozen in liquid nitrogen and stored at -80°C until subsequent analysis. A far too low number of subjects consented to have muscle biopsies taken at 6M. Therefore, muscle analyses were restricted to the 0 and 3M time points. Twenty-four hour urine samples were collected at baseline and after 3M and 6M into a plastic container and a 5 mL aliquot was stored at -20°C.

Quantification of 25-Hydroxyvitamin D

25-Hydroxyvitamin D was measured in serum samples of all participants using the Elecsys Vitamin D total assay (Cobas Instrument). Briefly, the assay kit employs a vitamin D binding protein as capture protein to bind vitamin D_3 and D_2.

Quantification of plasma and urinary compounds

Plasma creatine, creatinine, guanidinoacetate, carnitine and acetylcarnitine were measured by LC-MSMS with butanol-HCl derivatization. Urinary creatinine was measured using the Cobas Instrument (Roche). Plasma beta-alanine and urinary biomarkers for meat intake (pi-methyl-histidine, tau-methylhistidine and anserine) were measured by HPLC. Plasma and urine of all participants at 3 time points was deproteinized using 35 % sulfosalicylic acid (SSA) and centrifuged (16,000 g for 5 min). Two-point six µL of deproteinized supernatant was mixed with 77.4 µL of AccQ Fluor Borate buffer and 20 µL of reconstituted Fluor Reagent from the AccQTag chemistry kit (Waters). The same method was applied to the combined standard solutions of beta-alanine (Sigma) for plasma, and pi-methyl-histidine (Sigma), tau-methyl-histidine (Sigma) and anserine (Sigma) for urine. The derivatized samples were applied to a Waters HPLC system with following parameters: AccQTag column (3.9 x 150 mm, 5 µm) for beta-alanine quantification and Xbridge BEH column (4.6 x 150mm, 2.5µm) for pi-methyl-histidine, tau-methyl-histidine and anserine quantification, both with fluorescence detector (excitation/emission wavelength: 250/395 nm). The column was equilibrated with buffer A [10% eluent A (Waters) – 90% H_2O], buffer B (100% acetonitrile) and buffer C (100% H_2O) at a flow rate of 1ml/min at room temperature.

Quantification of muscle carnitine and creatine

Liquid nitrogen frozen wet muscle samples were freeze-dried before being powdered and checked for all visible blood and connective tissue, which were removed under low grade microscopy. Then muscle metabolites were extracted by adding cold 0.5 mM perchloric acid (PCA, containing 1 mM EDTA) to the muscle powder while on ice in a ratio 1 ml of PCA to every 12.5 mg of muscle powder. Then, samples were gently vortexed for 10 min before being centrifuged (10,000 rpm for 3 min at 4°C). Supernatants were then carefully removed to new test tubes and neutralised with 2.2 mM $KHCO_3$. The perchlorate precipitates were removed by centrifugation (10,000 rpm for 3 min at 4°C), and the supernatants (metabolite extracts) were removed to new test tubes and stored at -80C for subsequent analysis. Muscle PCA extracts were used to determine muscle creatine (Cr) and phosphocreatine (PCr) concentrations and free and acetylcarnitine concentrations employing spectrophotometric and radioactive methods described by Harris et al. (1974) and Cederblad et al. (1990), respectively. Muscle total creatine and carnitine concentrations were expressed as the sum of Cr and PCr, and the sum of free and acetylated carnitine, respectively.

Proton magnetic resonance spectroscopy

The carnosine levels in soleus and gastrocnemius muscles of all subjects at 0M, 3M and 6M was measured by proton magnetic resonance spectroscopy (^{1}H-MRS), as previously described (Baguet *et al.*, 2010*a*). The subjects were lying in supine position 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 with a 3 Tesla whole body MRI scanner (Siemens Trio, Erlangen) equipped with a spherical knee-coil. Single voxel point-resolved spectroscopy (PRESS) sequence with the following parameters was used: repetition time (TR) = 2.000 ms, echo time (TE) = 30 ms, number of excitations = 128, 1.024 data points, spectral bandwidth of 1.200 Hz, and a total acquisition time of 4.24 min. The average voxel size was 40 mm x 10 mm x 28 mm for soleus and 40 mm x 11 mm x 29 mm for gastrocnemius and the line width of the water signal was on average 25.2 Hz (soleus) and 28.3 Hz (gastrocnemius), following shimming procedures. The absolute carnosine content (in mM) was calculated as described before by Baguet et al. (2010*a*).

Statistics

A two-way mixed-model analysis of variance (ANOVA) was used to evaluate plasma and urinary metabolite concentrations, muscle carnosine, time to exhaustion (TTE) and VO_{2max} with 'group' (Veg+Suppl; Veg+Pla; control) as between-subject factor and 'time' (0M; 3M; 6M) as within-subject factor (SPSS statistical software, SPSS Inc, Chicago, USA). For the analysis of capillary lactate and pH, measurements before and after the incremental cycling test were included as another within factor (start; end). In case of significance, analyses were repeated for each group separately and pairwise comparisons were used to compare the different time points. A 3 x 2 repeated measures ANOVA was used to analyse muscle biopsy metabolite concentrations with 'group' (Veg+Suppl; Veg+Pla; control) as between-subject factor and 'time' (0M; 3M) as within-subject factor. In case of significance, pairwise comparisons were performed to compare 0 to 3 months for each group separately. Correlations between serum 25-Hydroxyvitamin D and plasma and muscle carnitine concentrations were obtained by means of Pearson correlations. Values are presented as mean ± SD and statistical significance threshold was set at $p \leq 0.05$.

RESULTS

Creatine metabolism

In order to evaluate the effect of a long-term vegetarian diet in previous omnivores on creatine metabolism, plasma creatine (Figure 1A), creatinine (Figure 1B) and guanidinoacetate (Figure 1C), urinary creatinine (Figure 1D) and muscle phosphocreatine (Figure 2A), creatine (Figure 2B) and total creatine (Figure 2C) were measured. For plasma creatine, a significant interaction effect ($p < 0.001$) was found (Figure 1A), demonstrating a decrease of 46% from baseline in the Veg+Pla group ($p = 0.008$) and an increase of 195% in the Veg+Suppl group ($p = 0.008$) after 6 months. Plasma creatinine ($p = 0.102$) and guanidinoacetate ($p = 0.554$) and urinary creatinine ($p = 0.373$) were not influenced by any intervention. As can be seen on Figure 2, a significant interaction effect ($p < 0.001$) was found for muscle total creatine. Adhering to a creatine-free vegetarian diet for 3 months caused a significant decline (14.6%) in muscle total creatine (153.5 ± 24.0 to 128.8 ± 11.6 mmol/kg dry weight; $p = 0.025$), while this was slightly increased in the Veg+Suppl and control groups ($p = 0.007$ and $p = 0.087$, respectively).

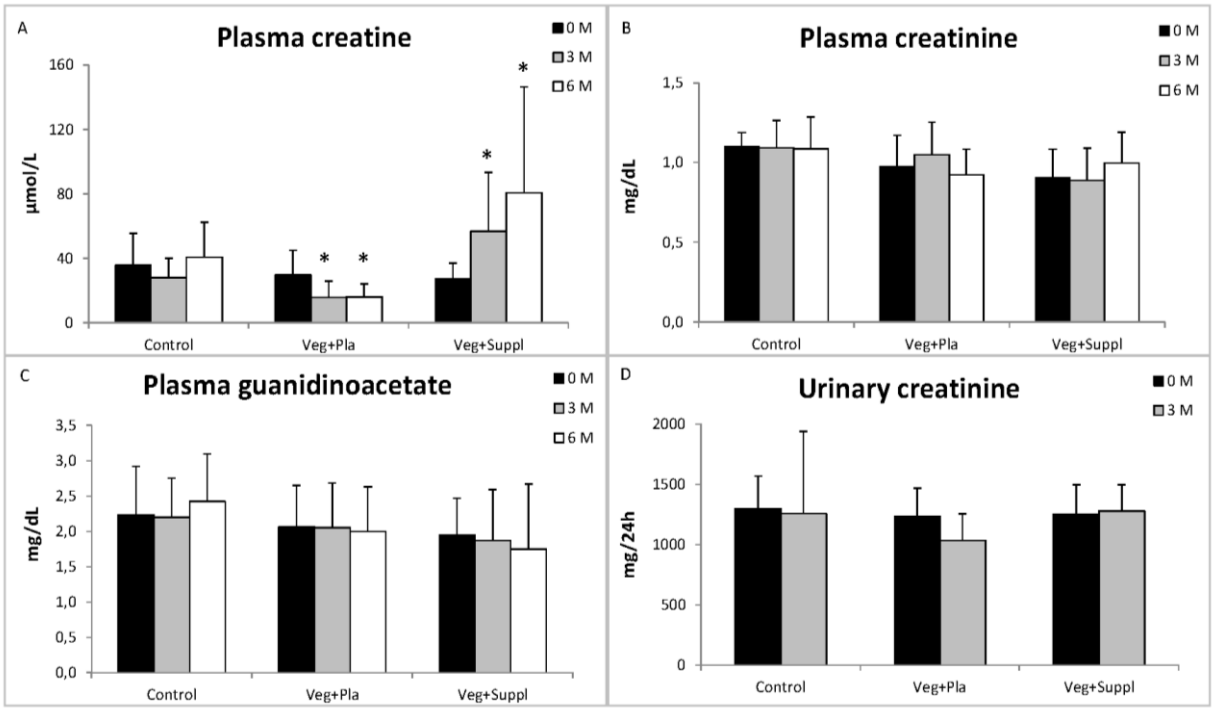

Figure 1. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on plasma creatine (A), plasma creatinine (B), plasma guanidinoacetate (C) and urinary creatinine (D) concentrations, analysed by a repeated measures ANOVA. 0M: baseline; 3M: 3 months; 6M: 6 months. * $p < 0.05$ compared to 0M

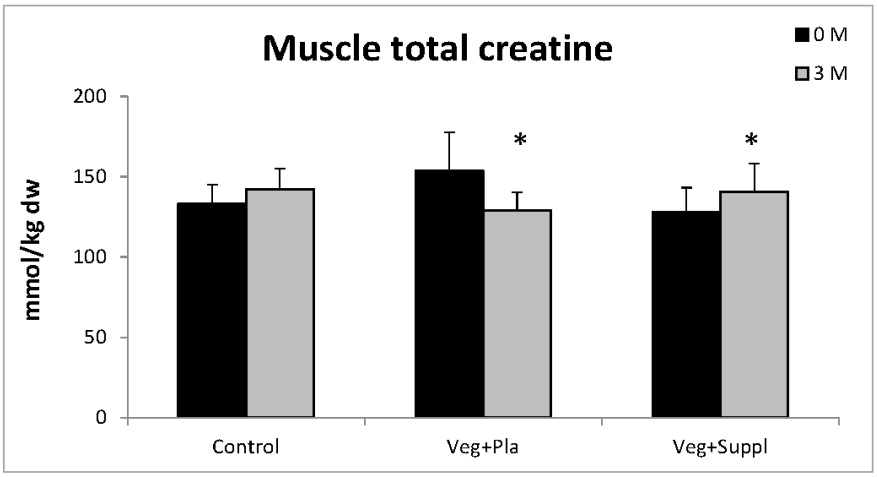

Figure 2. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on muscle total creatine concentrations analysed by a repeated measures ANOVA. 0M: baseline; 3M: 3 months; dw: dry weight. * $p < 0.05$ compared to 0M

Carnitine metabolism

Plasma and muscle free carnitine, acetylcarnitine and total carnitine (free + acetylcarnitine forms) concentrations were measured to investigate the effect of induced vegetarianism on the carnitine metabolism in previous omnivore subjects. No significant interaction effect was found for any of these parameters. However, as shown in Figure 3A-C and 4A-C, significant main effect of time for plasma carnitine ($p = 0.003$), acetylcarnitine ($p = 0.002$), and total carnitine ($p = 0.001$), and muscle carnitine ($p = 0.049$) and total carnitine ($p = 0.001$) were found. As most of carnitine parameters seem to decline independently from intervention, but rather as a result of a seasonal pattern, especially towards the 3M time point that coincided with the end of the winter, we decided, therefore, to evaluate if carnitine seasonal pattern could be related to the vitamin D status.

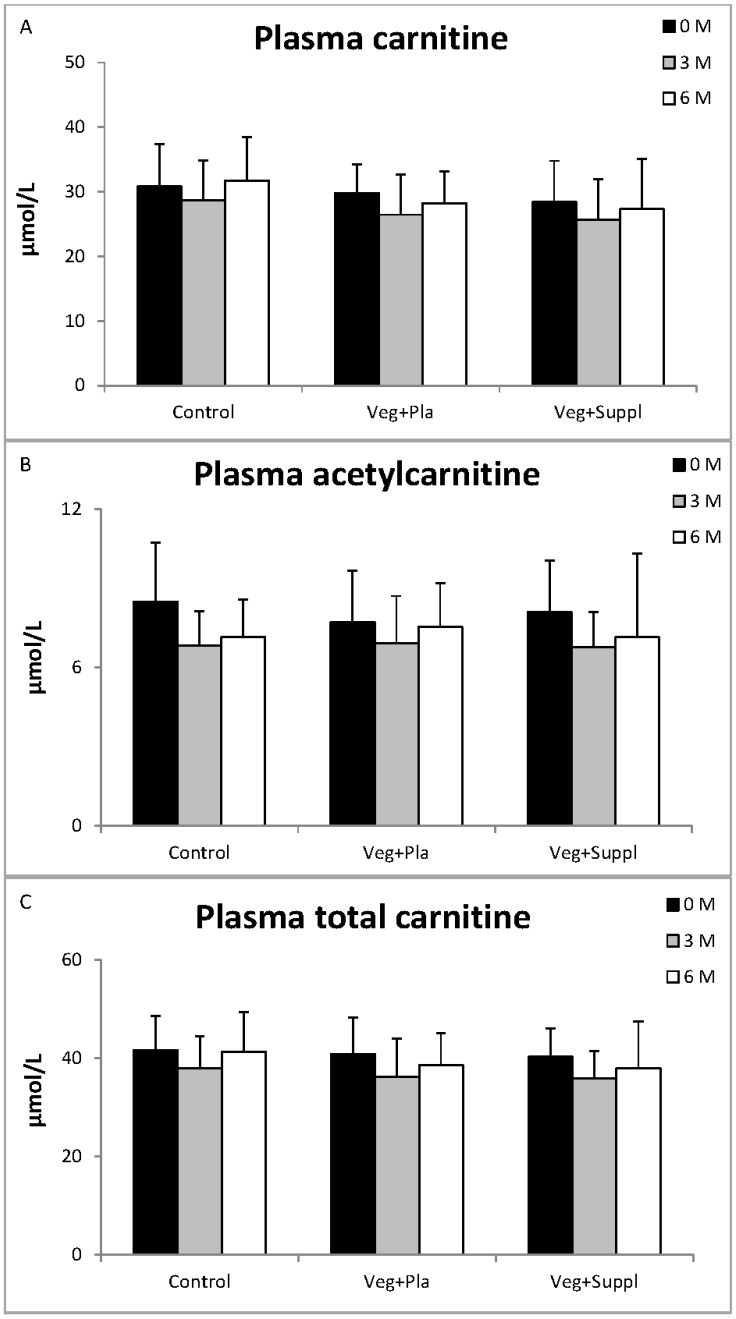

Figure 3. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on plasma carnitine (A), plasma acetylcarnitine (B) and plasma total carnitine (C) concentrations. 0M: baseline; 3M: 3 months; 6M: 6 months.

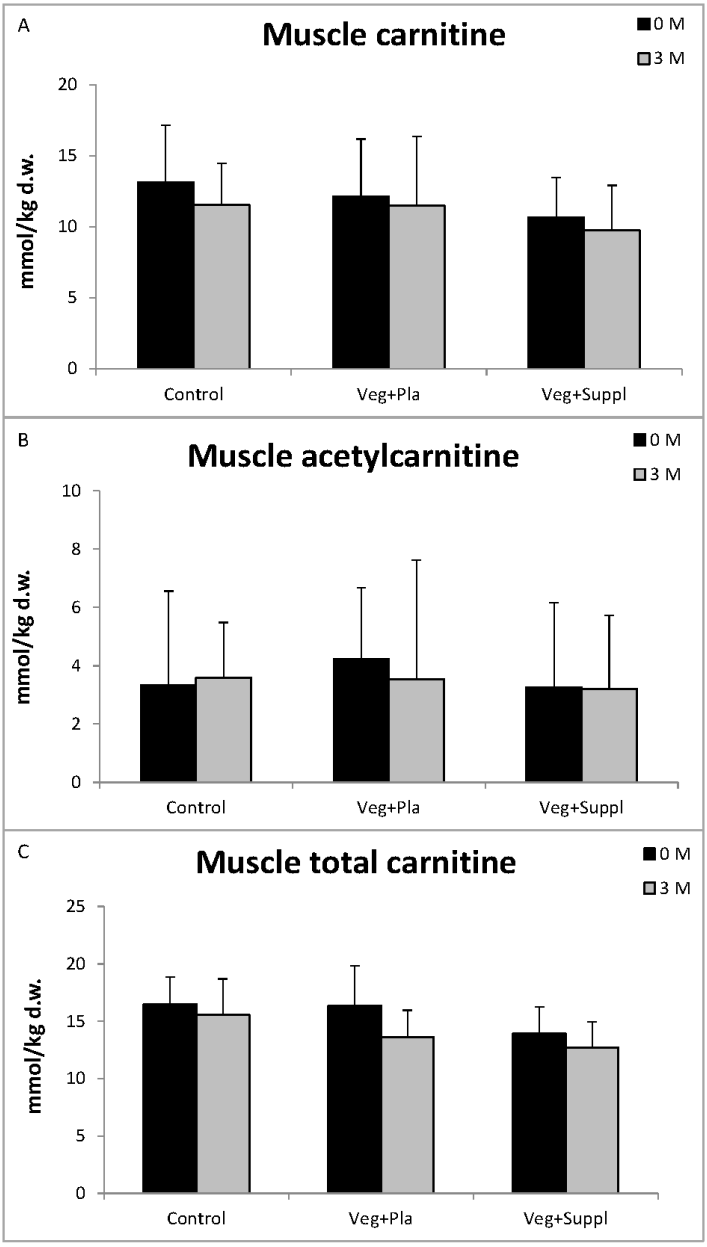

Figure 4. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on muscle carnitine (A), muscle acetylcarnitine (B) and muscle total carnitine (C) concentrations. 0M: baseline; 3M: 3 months; dw: dry weight.

Serum 25-Hydroxyvitamin D

Similar to most of the carnitine forms, a significant main effect of time ($p < 0.001$) for serum 25-Hydroxyvitamin D concentration was found, exemplified by a significant decrease from 0 to 3M and a return to baseline at 6M (Figure 5A). To check whether the 25-Hydroxyvitamin D levels were the underlying mechanism for the observed pattern of carnitine levels in plasma and muscle, correlations between these parameters were explored. Surprisingly, a significant negative rather than positive correlation between

serum 25-Hydroxyvitamin D levels and plasma total carnitine (r = -0.34, p < 0.001) (Figure 5B) was observed. No significant correlation between 25-Hydroxyvitamin D levels and total muscle carnitine was observed (r = -0.104, p = 0.422). After clustering the subjects in vitamin D-deficient (<25 ng/ml at 0 or 3M) and non-deficient (>25 ng/ml) groups, significant higher total plasma carnitine levels in the non-deficient than in the deficient group were found at each time point (0M p = 0.013; 3M p = 0.064; 6M p = 0.002). 25-Hydroxyvitamin D deficient subjects also displayed a significantly lower decrease in vitamin D levels from 0 to 3M compared to non-deficient subject (-4.53 ng/ml vs 8.50 ng/ml, p = 0.014).

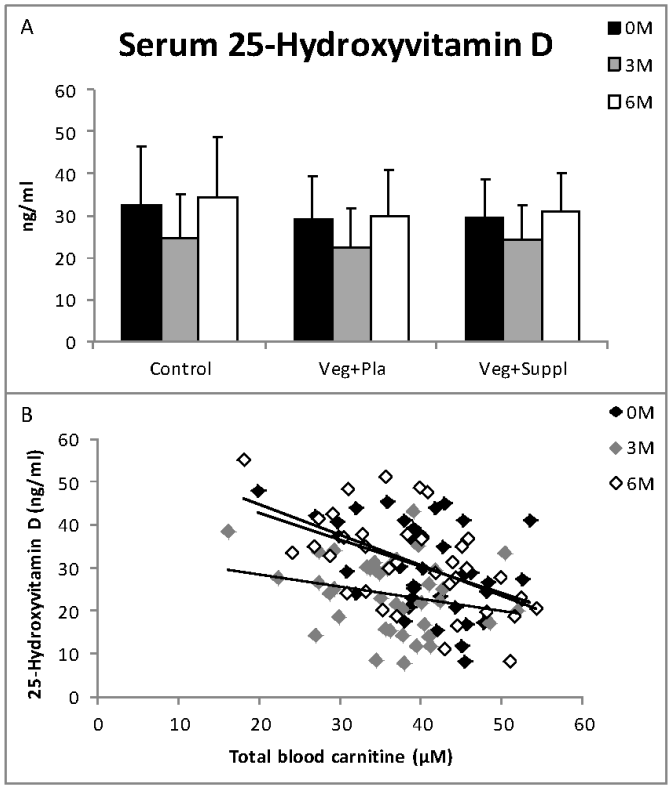

Figure 5. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on serum 25-Hydroxyvitamin D concentrations (A) and correlations between 25-Hydroxyvitamin D and total plasma carnitine content at 0M (r = -0.432, p = 0.008), 3M (r = -0.24, p = 0.153) and 6M (r = -0.561, p ≤ 0.001) (B). 0M: baseline; 3M: 3 months; 6M: 6 months.

Carnosine metabolism

Fasting plasma beta-alanine concentrations remained stable throughout the 6-month intervention period in the Veg+Pla and control group, but not in the Veg+Suppl group (Figure 6A). The latter group showed a significant increase in plasma beta-alanine concentrations after 3 months of vegetarian diet combined with 0.8 g beta-alanine per

day (+27.2%; p = 0.027), with no further increase in the following 3 months when the daily dose of beta-alanine was lowered to 0.4 g/day (p = 0.740 vs 3 months). A significant (p < 0.001) interaction effect (time x group) was found for soleus muscle carnosine content (Figure 6B). In accordance with plasma beta-alanine, muscle carnosine did not change over time in the Veg+Pla group (p = 0.619) nor in the control group (p = 0.790). When a 6-month vegetarian diet was combined with beta-alanine supplementation (Veg+Suppl group), soleus carnosine content increased by 26% (p < 0.001). This increase was already established after 3 months (+28%; p < 0.001), with no further increase in the subsequent 3 months. For gastrocnemius muscle, similar results were found (Figure 6C); a significant increase in the Veg+Suppl group of 28% and 41% after 3 (p = 0.009) and 6 (p = 0.001) months respectively, while carnosine remained stable in the Veg+Pla (p = 0.275) and control group (p = 0.293).

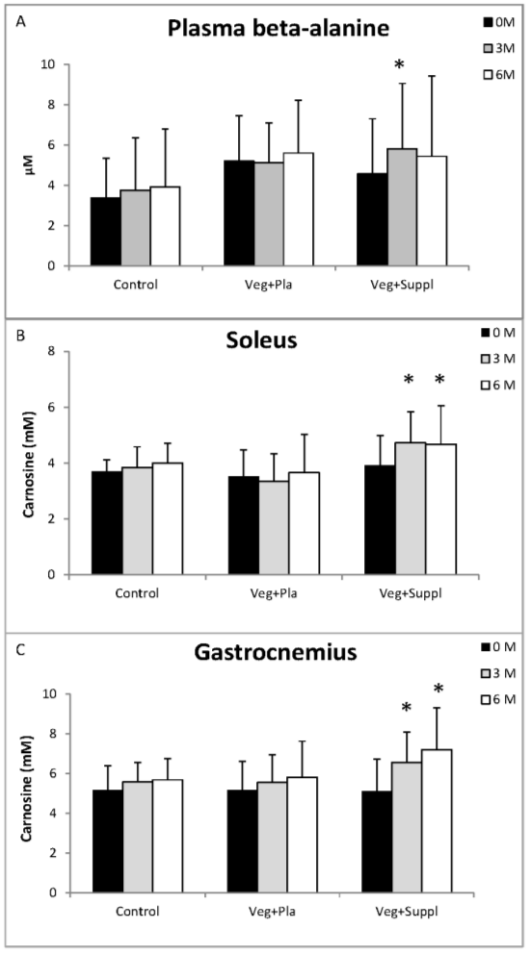

Figure 6. Effect of an omnivorous diet (Control), a vegetarian diet (Veg+Pla) and a vegetarian diet combined with creatine and beta-alanine supplements (Veg+Suppl) on plasma beta-alanine (A), soleus carnosine (B) and gastrocnemius carnosine (C) concentrations. 0M: baseline; 3M: 3 months; 6M: 6 months. * p < 0.05 compared to 0M

Compliance control via urinary biomarkers for meat intake

Both dietary meat intake urinary markers pi-methyl-histidine and anserine were significantly lower at 6M than at 0M in the Veg+Pla and Veg+Suppl groups (pi-methyl-histidine: p = 0.006 and p = 0.004, anserine: p = 0.075 and p = 0.048, respectively), but not in the control group (pi-methylhistidine: p = 0.411; anserine: p = 0.526) (Table 1). Although urinary tau-methyl-histidine excretion at 6M was significantly lower than at 0M in all experimental groups, the magnitude of this decline was more pronounced in the Veg+Pla and Veg+Suppl groups than in control.

Table 1. Urinary concentrations of pi-methyl-histidine, tau-methyl-histidine and anserine for the 3 experimental groups at 0M, 3M and 6M analysed by a repeated measures ANOVA [1,2]

		0M	3M	6M
Pi-methyl-histidine (mg/24h)	Control	161.9 ± 111.2	117.8 ± 124.8	122.5 ± 168.8
	Veg+Pla	167.4 ± 145.3	26.4 ± 10.7*	15.1 ± 4.7*
	Veg+Suppl	212.9 ± 201.0	28.7 ± 9.1*	18.0 ± 6.7*
Tau-methyl-histidine (mg/24h)	Control	55.2 ± 25.3	74.6 ± 61.6	42.6 ± 21.5*
	Veg+Pla	63.1 ± 20.0	43.1 ± 15.3*	27.6 ± 7.1**
	Veg+Suppl	63.0 ± 22.3	46.1 ± 16.0*	26.5 ± 8.5**
Anserine (mg/24h)	Control	15.0 ± 14.3	9.5 ± 7.4	11.6 ± 23.2
	Veg+Pla	9.2 ± 7.9	5.5 ± 2.9	4.0 ± 1.9$
	Veg+Suppl	10.7 ± 12.9	4.4 ± 2.2$	2.6 ± 1.1*

[1] Data are mean ± SD. 0M: baseline; 3M: 3 months; 6M: 6 months; Veg+Pla: Vegetarian+Placebo; Veg+Suppl: Vegetarian+Supplemental creatine and beta-alanine

[2] * p<0.05 compared to 0M; ** p<0.001 compared to 0M; $ 0.05<p<0.10 compared to 0M

Incremental cycling test

VO2max and time to exhaustion (TTE) did not differ between the experimental groups at baseline, nor did it change during the 6-month intervention period (Table 2). Blood lactate increased to a range of 11-14 mmol/l by the end of cycling. Blood pH at rest was approximately 7.41-7.42 and was not affected by the subsequent intervention (vegetarian/supplementation). The incremental cycling test elicited a marked acidosis (pH 7.23-7.29) in all intervention groups (Table 3). These parameters (VO2max, TTE, lactate and pH) showed neither a significant intervention nor time or interaction effect.

Table 2. VO_{2max} and TTE in an incremental cycling exercise test for the 3 experimental groups at 0M, 3M and 6M analysed by a repeated measures ANOVA[1]

		0M	3M	6M
VO_{2max} (ml/min/kg)	Control	40.4 ± 6.4	39.0 ± 8.8	39.4 ± 6.9
	Veg+Pla	39.4 ± 6.4	37.8 ± 8.0	38.1± 6.6
	Veg+Suppl	36.6 ± 6.3	34.8 ± 4.7	34.5 ± 4.9
TTE (min)	Control	14.5 ± 1.9	14.0 ± 3.0	14.6 ± 2.2
	Veg+Pla	15.1 ± 2.7	14.7 ± 2.5	15.0 ± 2.5
	Veg+Suppl	13.8 ± 2.6	13.6 ± 2.4	13.8 ± 2.9

[1] Data are mean ± SD. 0M: baseline; 3M: 3 months; 6M: 6 months; TTE: time to exhaustion; Veg+Pla: Vegetarian+Placebo; Veg+Suppl: Vegetarian+Supplemental creatine and beta-alanine

Table 3. Capillary lactate and pH at 0M, 3M and 6M at rest and at the end of the incremental cycling test[1]

		0M		3M		6M	
		Start	End	Start	End	Start	End
Lactate (mmol/l)	Control	1.9 ± 0.5	10.7 ± 1.6	2.4 ± 1.0	11.4 ± 2.6	2.0 ± 0.5	12.1 ± 2.0
	Veg+Pla	2.0 ± 0.7	12.4 ± 4.2	2.0 ± 0.9	14.1 ± 2.1	1.7 ± 0.4	12.7 ± 1.1
	Veg+Suppl	1.8 ± 0.8	10.7 ± 2.7	2.2 ± 0.9	12.2 ± 2.3	2.2 ± 1.1	13.1 ± 2.1
pH	Control	7.40 ± 0.02	7.28 ± 0.03	7.40 ± 0.03	7.27 ± 0.04	7.42 ± 0.04	7.29 ± 0.05
	Veg+Pla	7.41 ± 0.02	7.24 ± 0.07	7.42 ± 0.03	7.23 ± 0.04	7.43 ± 0.02	7.27 ± 0.03
	Veg+Suppl	7.42 ± 0.02	7.28 ± 0.06	7.41 ± 0.01	7.26 ± 0.04	7.43 ± 0.01	7.28 ± 0.04

[1] Data are mean ± SD. 0M: baseline; 3M: 3 months; 6M: 6 months;Veg+Pla: Vegetarian+Placebo; Veg+Suppl: Vegetarian+Supplemental creatine and beta-alanine

DISCUSSION

The present study demonstrates that whole body creatine homeostasis was disturbed by a 3-month vegetarian diet in omnivorous women, while carnosine and carnitine homeostasis was not affected. The measurements of muscle carnosine demonstrate that carnosine homeostasis is even not affected after 6 months of vegetarianism, but no muscle data are available at this timepoint for creatine and carnitine. However, the plasma measurements suggest that carnitine homeostasis is maintained even at 6 months, while creatine homeostasis is not. Total creatine is, alongside glycogen, the most abundant metabolite in human skeletal muscle with concentrations around 120-150 mmol/kg dry weight (dw), and is known to have a high turnover rate, as 1.7% of the total body creatine pool is daily non-enzymatically converted into creatinine. Consequently, in the women participating in this study, containing ~110 g of total creatine, ~1.6 g/day is roughly converted into creatinine and has to be replaced by creatine from diet or from *de novo* biosynthesis (23). It has been demonstrated that the average creatine consumed in a diet containing meat and fish is 1.0 g/d (30) and 1-2 g/day of creatine is endogenously synthesized from arginine, glycine and methionine in liver, kidney and pancreas (23). Thus, in case the diet is almost free of creatine, approximately twice as much creatine needs to be synthesized in the body to replace the amount of creatine irreversibly degraded to creatinine. The current data demonstrate that endogenous creatine synthesis cannot fully compensate for the lack of dietary intake in a vegetarian context. When muscle creatine stores decline in response to a vegetarian diet, one would also expect that urinary creatinine declines to a similar degree, as there is no enzymatic control for the hydrolysis of creatine and total muscle mass is assumed to remain stable. Muscle total creatine and urinary creatinine declined by 14.6% and 14.2% respectively in the Veg+Pla group (3M), although the former reached statistical significance (interaction effect $p < 0.001$) and the latter did not (interaction effect $p = 0.373$).

Carnitine and carnosine have a lower muscular concentration (20-30 mmol/kg dw) and a much slower turnover rate than creatine. Healthy subjects excrete carnitine at a rate of 5 µmol/kg/day, which, for the women participating in this study, is 315 µmol/day (~0.05 g) (7). For muscle carnosine, wash-out time after a period of carnosine loading by beta-alanine supplementation was shown to be a slow process that takes 6-20 weeks (25),

demonstrating the slow degradation rate of carnosine. Baguet and colleagues (25) calculated that, in absolute terms, the elimination of carnosine is ~0.5 g/day and is thus 3 to 4-fold slower than that for creatine (1.6 g/day).

To our knowledge, this is the first long-term intervention study demonstrating that body creatine stores decline after a vegetarian diet. Our results are in accordance with cross-sectional data on plasma and urinary creatine and creatinine values in vegetarian subjects (17). Muscle total creatine decreased within 3 months by 14% in our subjects, which is in accordance with the cross-sectional decrease (-10%) in muscle total creatine found in the study of Burke et al. (20) and the longitudinal decrease (-10%) after a 3-week intervention period in omnivorous subjects (24). However, this decrease did not affect VO_{2max} nor TTE of the incremental cycling test, although it might affect other short-term high-intensity performances such as sprinting, in which phosphocreatine plays a key role.

Interestingly, creatine supplementation combined with a vegetarian diet was able to maintain muscle total creatine content at baseline levels after 3 months and even triple plasma creatine concentrations at 6 months, thereby making creatine a proper supplement for vegetarian athletes participating in high-intensity exercises. A daily dose of 1 g creatine monohydrate seems to be a sufficient dose to prevent deficiencies, which is considerably lower than the 3-20 g per day required to elevate muscle creatine stores above the baseline set point (31). As the daily ingestion of 0.88 g creatine (1 g creatine monohydrate) is adequate to avoid a decline in body creatine stores on the background of a creatine-free vegetarian diet, the remainder of the daily creatine requirement is probably met by endogenous synthesis in kidney and liver.

Carnitine homeostasis of either plasma or muscle was not influenced by a vegetarian diet. Dietary carnitine intake is mainly attributable to the intake of meat and fish (animal source), although it is not completely absent in dairy products and some vegetables, fruit and cereals (32). Thus, carnitine is also present in moderate amounts in a vegetarian diet and has a slow turnover rate, which is in line with the absence of an effect of 6 months vegetarianism on plasma carnitine concentrations, as we hypothesized. As all experimental groups display the same pattern in the current study, namely lower carnitine and acetylcarnitine concentrations at 3M, which seemed to be restored at 6M,

this may reflect seasonal variations rather than effects of the intervention. The study was initiated in mid-December and the intermediate measurements were performed in March, thus the first half of the intervention period was during winter, while the second part, with the final measurements in the second half of June, was during spring. Therefore, we hypothesized that vitamin D status may be an underlying mechanism for the observed seasonal fluctuations in carnitine homeostasis. This hypothesis was based on the findings of Dursun et al. (33) who found decreased plasma carnitine levels and increased carnitinuria in severe vitamin D-deficiency patients (rickets), and rodent studies (34) have documented that low vitamin D status impairs lipid oxidation capacity, including carnitine palmitoyltransferase-1 (CPT-1) expression. Our results indicate that similar to carnitine, seasonal fluctuations are found in serum vitamin D concentration on group level. Yet, when looking at individual level, a significant *negative* correlation between serum vitamin D and plasma total carnitine content was found, which would indicate that vitamin D cannot explain the seasonal fluctuations in carnitine homeostasis.

Published cross-sectional data showed lower plasma carnitine concentrations in vegetarians than in omnivores (16–19), which is in contrast to our present data. However, this could be accounted for by difference in the length of vegetarianism (1.5 - 20 yrs vs the present 6 months). It is hypothesized that renal conservation mechanisms and thus higher carnitine reabsorption may occur in vegetarians to compensate for the lower carnitine concentrations obtained from the diet, thereby keeping plasma carnitine concentrations within a relatively normal range (18,35). Furthermore, some inconsistency exists also regarding muscle carnitine content, as Novakova et al. (16) did not find decreased content in vegetarians, while Stephens et al. (19) reported a 17% reduction. However, it should be noted that the study of Novakova et al. (16) included only male vegetarians that consumed a vegetarian diet for at least 1.5 yrs, while the study of Stephens et al. (19) included both male and female subjects who are vegetarians for 11 years on average. It can be concluded that seasonal variations probably have a bigger impact on body carnitine homeostasis than vegetarian dietary habits, although it cannot be excluded that carnitine stores could be affected by long-lasting vegetarianism (>1.5 yrs).

Given that beta-alanine is the rate-limiting precursor for carnosine synthesis in human muscle cells and vegetarian diets are free of beta-alanine and carnosine (including its methylated variants anserine and balenine), it is somewhat surprising that muscle carnosine content was unaltered after a 6-month vegetarian diet in previously omnivorous subjects. This suggests that maintenance of carnosine homeostasis does not depend on the nutritional supply of beta-alanine. Thus, endogenous beta-alanine synthesis can probably entirely compensate for the absence of dietary beta-alanine and thereby maintain body homeostasis, at least during the first months of vegetarianism. Current findings on muscle carnosine content are somewhat in contrast with cross-sectional data on long-term vegetarians (>7yrs) in the study of Harris et al. (22) and Everaert et al. (21). However, the study of Harris et al. (22) included only 6 vegetarians, was not gender- and age-matched (which is important for carnosine; (36)) and was only published as a conference abstract. In the study of Everaert et al. (21), the lower muscle carnosine content in vegetarians (n=12) vs omnivores (n=38) only reached significance in the gastrocnemius, but not the soleus and tibialis anterior muscles.

Interestingly, a low dose of beta-alanine supplementation (0.8 g/d) for 3 months in vegetarians significantly increased plasma beta-alanine and muscle carnosine content by 20-30%. Knowing that the average daily intake of beta-alanine from an omnivore Western diet has been calculated to amount to ~330 mg/day (21), the daily intake of an additional ~500 mg/d, which corresponds to a total ingested dose of ~46 g in 3 months, is responsible for this increase. Because of the increase in carnosine above baseline concentration with this initial dose (0.8g/d), beta-alanine dose was reduced to 0.4g/day during the last 3 months of the study. This dose did not further enhance plasma beta-alanine and muscle carnosine concentrations, neither did it restore these concentrations to baseline, suggesting this dose was still higher than the normal dietary beta-alanine intake of these subjects.

Some urinary biomarkers of meat intake are known, such as pi-methyl-histidine, tau-methyl-histidine and anserine (37–39). A clear reduction in urinary excretion of these compounds, for pi-methyl-histidine to an order of magnitude lower than the original value, was observed in the vegetarian subjects proving that they complied with the vegetarian intervention. A limitation of the current study is that data of the muscle

biopsies at 6M are missing, making it impossible to draw conclusions on the effect of a 6-month vegetarian diet on intramuscular creatine and carnitine concentrations.

It can be concluded that body creatine stores decline by a 3-month vegetarian diet in omnivorous women and can be restored by creatine supplementation, which makes creatine a suitable carninutrient for supplementation in vegetarian athletes, especially those participating in resistance training and short-term, high-intensity exercise performances. Carnitine homeostasis was not disrupted by a 3-month vegetarian diet and carnosine homeostasis was not disrupted by a 6-month vegetarian diet, suggesting that endogenous synthesis can fully compensate to maintain these compounds at a homeostatic set point, making supplementation unnecessary in vegetarian subjects.

ACKNOWLEDGEMENT

The authors' responsibilities were as follows—LB, AB, TB, IE, SDH and WD: developed the study design; LB, AB, TB, IE, AV, CV and WD: performed and supervised the study intervention; LB, AB, TB, IE, AV, MP, SDH and WD: performed and supervised the data collection; LB, AB, TB, AV, IE, JD, EA, CTD, PG and WD: performed the data analyses and interpretation; LB, AB and WD: wrote the paper, is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; and all authors: provided intellectual input for the manuscript and read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

This study was financially supported by grants from the Research Foundation – Flanders (FWO G.0243.11 and G.0352.13N). Creatine supplements were kindly provided by Dr Ulrike Braun from AlzChem (Trostberg, Germany) and beta-alanine supplements were provided by Dr Roger Harris and Dr Zimin Liu from Natural Alternatives International (NAI, San Marcos, USA).

REFERENCES

Tantamango-Bartley Y, Jaceldo-Siegl K, Fan J, Fraser G. Vegetarian diets and the incidence of cancer in a low-risk population. Cancer Epidemiol Biomarkers Prev. 2013;22:286–94.

McEvoy CT, Temple N, Woodside J V. Vegetarian diets, low-meat diets and health: a review. Public Health Nutr. 2012;15:2287–94.

Orlich MJ, Singh PN, Sabaté J, Jaceldo-Siegl K, Fan J, Knutsen S, Beeson WL, Fraser GE. Vegetarian dietary patterns and mortality in Adventist Health Study 2. JAMA Intern Med. 2013;173:1230–8.

Leitzmann C. Vegetarian diets: what are the advantages? Forum Nutr. 2005;57:147–56.

McCarty MF. Sub-optimal taurine status may promote platelet hyperaggregability in vegetarians. Med Hypotheses. 2004;63:426–33.

Gualano B, Rawson ES, Candow DG, Chilibeck PD. Creatine supplementation in the aging population: effects on skeletal muscle, bone and brain. Amino Acids. 2016;1–13.

Steiber A, Kerner J, Hoppel CL. Carnitine: A nutritional, biosynthetic, and functional perspective. Mol Aspects Med. 2004;25:455–73.

Fritz IB, Marquis NR. The role of acylcarnitine esters and carnitine palmityltransferase in the transport of fatty acyl groups across mitochondrial membranes. Proc Natl Acad Sci U S A. 1965;54:1226–33.

Seiler S, Koves T, Gooding J, Wong K, Stevens R, Ilkayeva O, Wittmann A, DeBalsi K, Davies M, Lindeboom L, et al. Carnitine Acetyltransferase Mitigates Metabolic Inertia and Muscle Fatigue During Exercise. Cell Metab. 2015;22:65–76.

Blancquaert L, Everaert I, Derave W. Beta-alanine supplementation, muscle carnosine and exercise performance. Curr Opin Clin Nutr Metab Care. 2015;18:63–70.

Hobson RM, Saunders B, Ball G, Harris RC, Sale C. Effects of β-alanine supplementation on exercise performance: a meta-analysis. Amino Acids. 2012;43:25–37.

Wall BT, Stephens FB, Constantin-Teodosiu D, Marimuthu K, Macdonald IA, Greenhaff PL. Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. J Physiol J Physiol J Physiol. 2011;5894:963–73.

Twycross-Lewis R, Kilduff LP, Wang G, Pitsiladis YP. The effects of creatine supplementation on thermoregulation and physical (cognitive) performance: a review and future prospects. Amino Acids. Springer Vienna; 2016;1–13.

Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. Physiol Rev. 2013;93:1803–45.

Pinto CL, Botelho PB, Pimentel GD, Campos-Ferraz PL, Mota JF. Creatine supplementation and glycemic control: a systematic review. Amino Acids. 2016;In press.

Novakova K, Kummer O, Bouitbir J, Stoffel SD, Hoerler-Koerner U, Bodmer M, Roberts P, Urwyler A, Ehrsam R, Krähenbühl S. Effect of l-carnitine supplementation on the body carnitine pool, skeletal muscle energy metabolism and physical performance in male vegetarians. Eur J Nutr. 2016;55:207–17.

Delanghe J, De Slypere J-P, De Buyzere M, Robbrecht J, Wieme R, Vermeulen A. Normal Reference Values for Creatine, Creatinine, and Carnitine Are Lower in Vegetarians. Clin Chem. 1989;35:1988–9.

Lombard KA, Olson L, Nelson SE, Rebouche CJ. Carnitine status of lactoovovegetarians and strict vegetarian adults and children. Am J Clin Nutr. 1989;50:301–6.

Stephens FB, Marimuthu K, Cheng Y, Patel N, Constantin D, Simpson EJ, Greenhaff PL. Vegetarians have a reduced skeletal muscle carnitine transport capacity. Am J Clin Nutr. 2011;94:938–44.

Burke DG, Chilibeck PD, Parise G, Candow DG, Mahoney D, Tarnopolsky M. Effect of Creatine and Weight Training on Muscle Creatine and Performance in Vegetarians. Med Sci Sports Exerc. 2003;35:1946–55.

Everaert I, Mooyaart A, Baguet A, Zutinic A, Baelde H, Achten E, Taes Y, De Heer E, Derave W. Vegetarianism, female gender and increasing age, but not CNDP1 genotype, are associated with reduced muscle carnosine levels in humans. Amino Acids. 2011;40:1221–9.

Harris RC, Jones G, Hill C, Kendrick IP, Boobis L, Kim C, Kim H, Dang VH, Edge J, Wise J. The Carnosine Content of Vastus Lateralis in Vegetarians and Omnivores. FASEB J. 2007;21:A944.

Wyss M, Kaddurah-Daouk R. Creatine and Creatinine Metabolism. Physiol Rev. 2000;80:1107–213.

Lukaszuk JM, Robertson RJ, Arch JE, Moore GE, Yaw KM, Kelley DE, Rubin JT, Moyna NM. Effect of creatine supplementation and a lacto-ovo-vegetarian diet on muscle creatine concentration. Int J Sport Nutr Exerc Metab. 2002;12:336–48.

Baguet A, Reyngoudt H, Pottier A, Everaert I, Callens S, Achten E, Derave W. Carnosine loading and washout in human skeletal muscles. J Appl Physiol. 2009;106:837–42.

Baguet A, Everaert I, De Naeyer H, Reyngoudt H, Stegen S, Beeckman S, Achten E, Vanhee L, Volkaert A, Petrovic M, et al. Effects of sprint training combined with vegetarian or mixed diet on muscle carnosine content and buffering capacity. Eur J Appl Physiol. 2011;111:2571–80.

Harris R, Hultman E, Nordesjö L. Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man

at rest. Methods and variance of values. Scand J Clin Lab Invest. 1974;33:109–20.

Cederblad G, Carlin JI, Constantin-Teodosiu D, Harper P, Hultman E. Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle. Anal Biochem. 1990;185:274–8.

Baguet A, Bourgois J, Vanhee L, Achten E, Derave W. Important role of muscle carnosine in rowing performance. J Appl Physiol. 2010;109:1096–101.

Balsom PD, Söderlund K, Ekblom B. Creatine in Humans with Special Reference to Creatine Supplementation. Sport Med. 1994;18:268–80.

Hultman E, Söderlund K, Timmons JA, Cederblad G, Greenhaff PL. Muscle creatine loading in men. J Appl Physiol. 1996;81:232–7.

Krajčovičová-Kudláčková M, Šimončič R, Béderová A, Babinská K, Béder I. Correlation of carnitine levels to methionine and lysine intake. Physiological Research. 2000. p. 399–402.

Dursun A. Carnitinuria in rickets due to vitamin D deficiency. Turk J Pediatr. 2000;42:278–80.

Park S, Kim DS, Kang S. Vitamin D deficiency impairs glucose-stimulated insulin secretion and increases insulin resistance by reducing PPAR-ɣ expression in nonobese Type 2 diabetic rats. J Nutr Biochem. Elsevier Inc.; 2016;27:257–65.

Rebouche J, Lombard A, Chenard A. Renal adaptation to dietary carnitine in humans. Am J Clin Nutr. 1993;58:660–5.

Baguet A, Everaert I, Achten E, Thomis M, Derave W. The influence of sex, age and heritability on human skeletal muscle carnosine content. Amino Acids. 2012;43:13–20.

Cross a. J, Major JM, Sinha R. Urinary Biomarkers of Meat Consumption. Cancer Epidemiol Biomarkers Prev. 2011;20:1107–11.

Dragsted LO. Biomarkers of meat intake and the application of nutrigenomics. Meat Sci. Elsevier Ltd; 2010;84:301–7.

Altorf-van der Kuil W, Brink EJ, Boetje M, Siebelink E, Bijlsma S, Engberink MF, van ’t Veer P, Tome D, Bakker SJL, van Baak MA, et al. Identification of biomarkers for intake of protein from meat, dairy products and grains: a controlled dietary intervention study. Br J Nutr. 2013;110:810–22.

Study 4

Gene expression of carnosine-related enzymes and transporters in human skeletal muscle: influence of chronic beta-alanine supplementation

Blancquaert L, Everaert I, Stegen S, Derave W

Unpublished

BACKGROUND

Chronic oral beta-alanine supplementation can elevate muscle carnosine (beta-alanyl-L-histidine) content and subsequently improve high-intensity exercise performance (Harris *et al.*, 2006; Hill *et al.*, 2007). However, the regulation of muscle carnosine levels is still poorly understood. The uptake of the rate-limiting precursor beta-alanine and the enzyme responsible for the synthesis of carnosine are thought to be key steps. The several steps and their respective enzymes and transporters that are possibly involved in metabolic pathways of carnosine and constituent amino acids include: beta-alanine transport (TauT, PAT1, ATB0,+), beta-alanine availability regulated by beta-alanine synthesis (GADL1 and uracil degradation) on the one hand, and beta-alanine degradation (GABA-T and AGXT2) on the other hand, L-histidine availability regulated by histidine decarboxylase (HDC), carnosine synthesis (CARNS) and carnosine degradation (CNDP1 and CNDP2) and carnosine and/or histidine transport (POT-family (proton-coupled oligopeptide transporters): PEPT1, PEPT2, PHT1 and PHT2).

The expression of carnosine synthase (CARNS), tissue carnosinase (CNDP2), peptide/histidine transporter 1 (PHT1), taurine transporter (TauT) and proton coupled amino acid transporter 1 (PAT1) was already demonstrated in human skeletal muscle, whereas serum carnosinase (CNDP1), beta-alanine transporter $ATB^{0,+}$ and the other members of the POT-family are not expressed in human muscle tissue (Everaert *et al.*, 2013*a*). The expression of beta-alanine transaminases GABA-T and AGXT2, histidine decarboxylase (HDC) and glutamate decarboxylase-like protein 1 (GADL1) was not explored in the study of Everaert et al. (2013*a*). GABA-T and AGXT2 are generally considered to be highly expressed in kidney and liver, and to a much lower extent in skeletal muscle. However, since beta-alanine is a free amino acid and its availability is decisive for carnosine synthesis, the presence of this pathway inside myocytes deserves more thorough investigation. Moreover, HDC can be an alternative metabolic pathway for intramyocellular L-histidine and thus might also affect carnosine metabolism. Its expression was already demonstrated in human skeletal muscle (Romero *et al.*, 2016), suggesting that histidine might be degraded in muscle cells. GADL1 can directly synthesize beta-alanine from aspartate by a decarboxylation reaction. Its expression is until now only confirmed in skeletal muscle of rodents and cattle. In order to get a better understanding

of the regulation of muscle carnosine homeostasis, this study aims to investigate the mRNA expression of carnosine-related enzymes in human muscle that are putative players in the metabolic pathways of carnosine, such as GABA-T, AGXT2 and GADL1.

Moreover, the study of Everaert et al. (2013*a*) explored the transcriptional events in mouse skeletal muscle in response to beta-alanine supplementation, which is a known stimulus for carnosine synthesis. Beta-alanine supplementation increased both TauT, CARNS, CNDP2 and GABA-T expression, suggesting that muscles increase beta-alanine utilization through both dipeptide synthesis (CARNS) and deamination (GABA-T) and further oxidation, in conditions of excess availability. To investigate whether the human muscle carnosine metabolism is affected in a similar way upon beta-alanine supplementation, this study aimed to investigate the effect of chronic beta-alanine ingestion on the mRNA expression of all carnosine-related enzymes and transporters in human skeletal muscle.

METHODS

Muscle samples of the published study of Stegen et al. (2013*a*) were subjected to the qPCR analysis of the present study. In this previous study, 34 subjects, both males (n=16) and females (n=18), were supplemented with either pure beta-alanine during or interspersed between meals or slow-release beta-alanine during meals (all three groups ingested 3.2g/day for 46 days). By differentiating the timing of beta-alanine intake between groups, this study demonstrated that beta-alanine supplementation is more effective when co-ingested with a meal. Furthermore, slow-release beta-alanine was shown to be equally effective toward muscle carnosine loading compared to pure beta-alanine.

The qPCR analysis of the present study was performed on the muscle biopsies of the male subjects of the study of Stegen et al. (2013*a*) (n=16, age: 19.5 ± 1.2 yr, body weight: 73.2 ± 8 kg). Thus, these 16 subjects were all supplemented with 3.2g beta-alanine per day for 46 days, but differed in timing of beta-alanine intake and beta-alanine form (pure or slow release). Before and after this supplementation period, muscle carnosine levels were measured in calf muscles by ^{1}H-MRS and a muscle biopsy was taken from the vastus lateralis muscle.

Muscle biopsy

Following local anaesthesia, the muscle sample was taken with a 14 Gauge true-cut biopsy needle (Bard Magnum Biopsy gun; Bard, Inc., New Jersey, USA). The samples were then immediately frozen in liquid nitrogen and stored at -80°C until subsequent HPLC and qPCR analysis.

Determination of carnosine content in calf muscles by ^{1}H-MRS

Carnosine content of all the subjects was measured by proton magnetic resonance spectroscopy (^{1}H-MRS) in soleus and gastrocnemius medialis muscles, as described by Stegen et al. (2013*a*). The average voxel size for the soleus and gastrocnemius muscle of the men included in this analysis was respectively 40 mm x 11 mm x 29 mm and 40 mm x 12 mm x 30 mm. The line width of the water signal for the soleus and gastrocnemius muscle was on average respectively 23.7 Hz and 26.8 Hz, following shimming procedures. The absolute carnosine content (in millimolar; mM) was calculated as described before by Baguet et al. (2010*a*).

Carnosine and taurine quantification by HPLC

Muslce carnosine and taurine levels were determined by high-performance liquid chromatography (HPLC) as previously described (Blancquaert *et al.*, 2016).

mRNA expression of carnosine-related enzymes and transporters

Total RNA from human muscle was isolated using the TriPure Isolation Reagent (Roche, Basel, Switzerland) followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA purity was assessed using the A_{260}/A_{280} ratio. Using a blend of oligo(dT) and random primers, 500 ng of RNA was reversed transcribed with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's instructions. Quantitative PCR was carried out on a Lightcycler 480 system (Roche) using an 8 μl reaction mix containing 3 μl of template cDNA, 300 nM forward and reverse primers and 4 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions comprised a polymerase activation at 95 °C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Primer sequences

(Table 1) of most genes of interest are available in the literature (Everaert *et al.*, 2013*a*). The primer sequence for GABA-T, AGXT2 and GADL1 were newly designed using Primer Express, version 3.0 (Applied Biosystems). Sequence specificity was confirmed using NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To control the specificity of amplification, data melting curves were inspected and PCR efficiency was calculated. Normalized gene expression values were calculated by dividing the relative gene expression values (calculated by the ΔCt method) for each sample by the expression values of the geometric mean of ATP50 and rpl19 as selected by GeNorm (Vandesompele *et al.*, 2002).

Table 1. Primers used in quantitative PCR analysis

Function	Gene symbol	Forward primer (5'-3') Reverse primer (5'-3')	Source
Carnosine synthesis	CARNS	GGC-GTC-AGC-AAG-AAG-TTC-GT CCG-GTG-CTC-TGT-CAT-GTC-AA	Everaert et al., 2013
Carnosine hydrolysis	CNDP2	TTG-CTG-ATG-GGC-TCT-TTG-GT TCG-ATG-TCG-TCG-TAC-AGC-TTG-T	Everaert et al., 2013
Beta-alanine transport	TauT	CGT-ACC-CCT-GAC-CTA-CAA-CAA-A CAG-AGG-CGG-ATG-ACG-ATG-AC	Everaert et al., 2013
	PAT1	CAT-AAC-CCT-CAA-CCT-GCC-CAA-C GGG-ACG-TAG-AAC-TGG-AGT-GC	
Beta-alanine transaminase	GABA-T	CTG-GAG-ACG-TGC-ATG-ATT-AAC-C GTC-GCT-AAG-CAA-CCC-ATG-GT	Everaert et al., 2013
Beta-alanine synthesis	GADL1	GCC-ATT-AAG-GAG-AGG-ATG-ATG-AA GGC-GGA-AGA-AGT-TGA-CCT-TTC	Primer Express
Histidine transport	PHT1	GGT-TAT-GCG-ATC-CCC-ACT-GT ATC-AGG-AGG-CTT-GGT-GAT-GAA	Everaert et al., 2013
Reference genes	ATP50	GG-CCT-CCT-GTT-CAG-GTA-TAC-G CTT-GCT-CCA-GCT-TAT-TCT-GTT-TTG	RTprimerDB
	Rpl19	CGC-TGT-GGC-AAG-AAG-AAG-GTC GGA-ATG-GAC-CGT-CAC-AGG-C	RTprimerDB

Statistics

To investigate the changes in muscle carnosine and taurine content and the changes in mRNA expression of the carnosine-related enzymes and transporters after the chronic beta-alanine supplementation, a paired sample T-test was used. To test whether the form and timing of beta-alanine intake (pure or slow-release, with or without meal) differentially influences mRNA expression profiles, a repeated measures ANOVA was performed, with time (pre; post) als within factor and group (pure; pure+meal; slow-release+meal) as between factor. In case of significance, analyses were repeated for each group separately and a paired sample T-test was used to compare the different time points. All analyses were done with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p \leq 0.05$.

PRELIMINARY RESULTS AND CONCLUSIONS

Carnosine content, CARNS and CNDP2 expression

Beta-alanine supplementation increased muscle carnosine content in all investigated muscles. The carnosine concentrations measured by ^{1}H-MRS in soleus and gastrocnemius muscles were increased by 49.4% (4.08mM to 5.95mM, $p < 0.001$) and 31.2% (5.57mM to 7.16mM, $p < 0.001$), respectively (Fig 1). The carnosine content in vastus lateralis measured by HPLC showed a similar increase (+48.9%, $p < 0.001$) (data not shown).

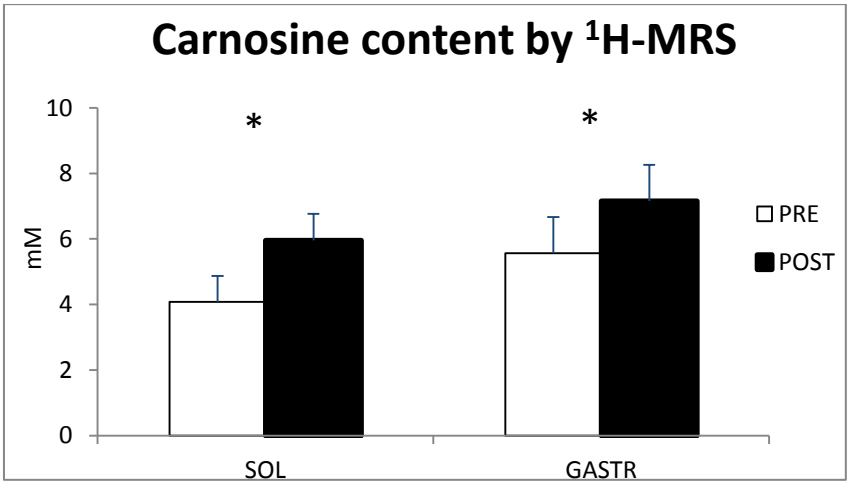

Figure 1. Carnosine content in soleus and gastrocnemius muscle before and after chronic beta-alanine supplementation.

* $p < 0.05$

Both CARNS (+175.2%, p = 0.01) and CNDP2 (+201%, p < 0.001) mRNA expression were significantly increased by chronic beta-alanine ingestion (Fig 2). This is in accordance with the results found in mice (Everaert *et al.*, 2013*a*) and illustrates that elevated plasma beta-alanine concentrations result in increased intramyocellular dipeptide synthesis. Whether CNDP2 is able to actively degrade carnosine in skeletal muscle is doubtful since the optimal pH for carnosine hydrolysis by CNDP2 has been shown to be 9.5 (Lenney *et al.*, 1985). Although CNDP2 is clearly upregulated by beta-alanine supplementation, it is generally believed that carnosine degradation in skeletal muscle (pH 7.1) by CNDP2 is minimal.

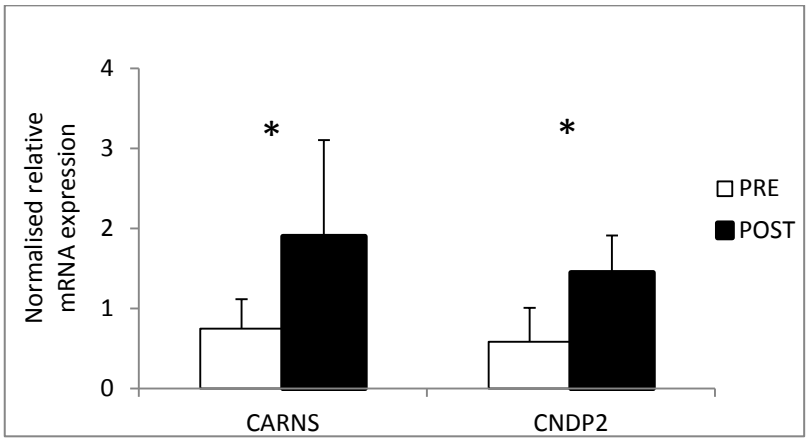

Figure 2. Effect of chronic beta-alanine supplementation on CARNS and CNDP2 mRNA expression. * p < 0.05

Taurine content, TauT and PAT1 expression

The vastus lateralis taurine content was not affected by chronic beta-alanine supplementation (Fig 3), suggesting that transsarcolemmal taurine uptake is not significantly suppressed by elevated plasma beta-alanine levels.

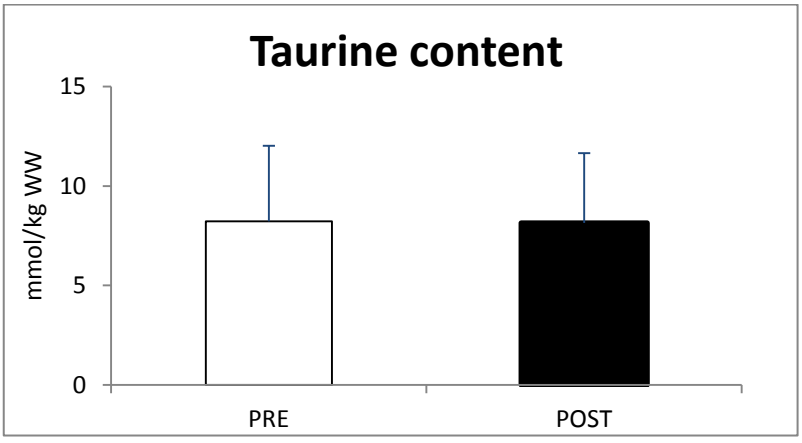

Figure 3. Taurine content in vastus lateralis muscle before and after chronic beta-alanine supplementation.

Chronic beta-alanine supplementation increased the gene expression of both TauT and PAT1 transporters (+132.6%, p = 0.084 and +198.0%, p = 0.05, respectively). When comparing the gene expression between the three groups (pure; pure+meal; slow-release+meal), TauT and PAT1 were only significantly upregulated in the slow-release+meal group (p = 0.016 and p = 0.01, respectively), but not in the groups that ingested pure beta-alanine. In mice, only TauT was modified by beta-alanine supplementation, suggesting that TauT is, albeit not the only, yet probably the most dominant transporter for the uptake of beta-alanine in mice skeletal muscle. As both transporters are upregulated by beta-alanine supplementation in humans, this might be an indication that they are both involved in the process of transsarcolemmal beta-alanine uptake. The upregulation of TauT might also be a mechanism to maintain intramyocellular taurine levels following increased circulating beta-alanine levels (thus taurine uptake is not negatively affected by increased beta-alanine uptake).

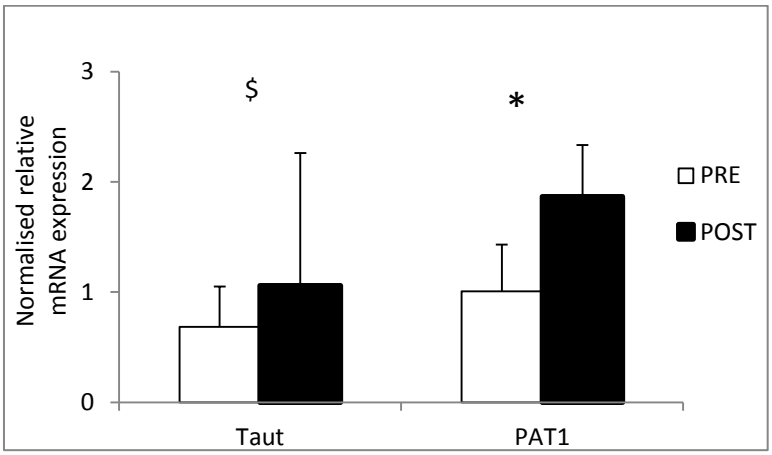

Figure 4. Effect of chronic beta-alanine supplementation on mRNA expression of beta-alanine transporters TauT and PAT1. * p < 0.05, $ 0.05 < p > 0.1

Beta-alanine availability: GABA-T, AGXT2 and GADL1 expression

GADL1, a putative pathway for intramyocellular beta-alanine synthesis, is for the first time shown to be expressed in human skeletal muscle. In contrast to hypothesized, the mRNA expression is significantly increased (+77%, p = 0.004) upon beta-alanine supplementation (Fig 5). Because GADL1 is also able to synthesize taurine, this upregulation might be a strategy to maintain taurine levels upon beta-alanine supplementation. It remains to be investigated whether GADL1 is actually active inside muscle cells and to what extent its contribution to beta-alanine availability is noteworthy.

Moreover, we aimed to investigate the expression of beta-alanine transaminase GABA-T and AGXT2. GABA-T was shown to be expressed in human muscle, although the expression seemed to be very low (illustrated by high Ct values). Moreover, GABA-T expression was slightly but non-significantly decreased by beta-alanine supplementation (Fig 5), which is in contrast to the findigs in mice skeletal muscle (Everaert *et al.*, 2013*a*). For AGXT2, 2 primer pairs were tested but no proper primers could be found since the efficiency curve of the investigated primer pairs was not linear (Ct values were equally high for the different dilutions that were tested) or primers were not specific (as illustrated by double peaks in the melting curves). Based on this data, we can conclude that no suitable primers were found, and it might be suggested that AGXT2 is not or only very minimally expressed in human skeletal muscle and thus its contribution to beta-alanine oxidation is negligible. Nevertheless, both GABA-T and AGXT2 are known to be highly expressed in liver and kidney where they significantly contribute to beta-alanine oxidation, as demonstrated by Blancquaert et al. (2016). Thus, intramyocellular beta-alanine degradation is probably not a main pathway in the regulation of muscle carnosine homeostasis.

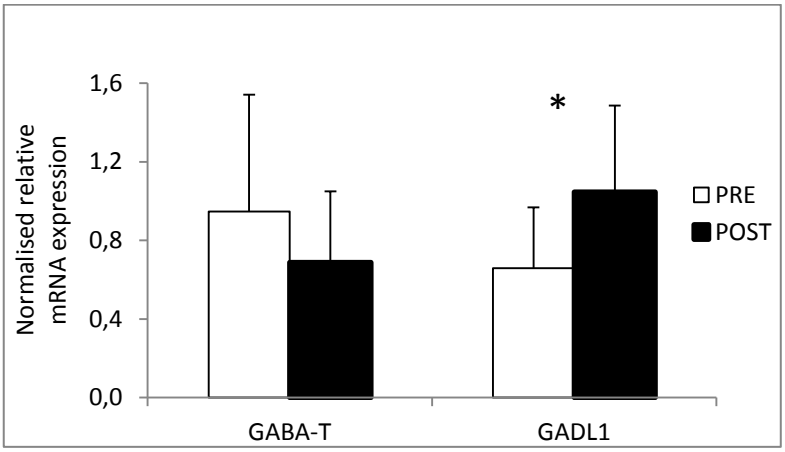

Figure 5. Effect of chronic beta-alanine supplementation on mRNA expression of GABA-T and GADL1. * $p < 0.05$

L-histidine transport: PHT1

PHT1, the histidine transporter that was shown to be expressed in human skeletal muscle, does not show altered expression in reponse to beta-alanine supplementation (normalised relative mRNA expression: 0.82 to 1.09, $p = 0.113$), which is in accordance with the results found in mice muscle. Thus, following beta-alanine supplementation, expression of the histidine transporter was not affected.

Summarizing these data on mRNA expression, we can conclude that, similar to mice, increased circulating beta-alanine levels stimulate the gene expression of TauT, PAT1 and CARNS, suggesting that, in conditions of excess availability, muscles increase beta-alanine uptake and utilization through dipeptide synthesis (CARNS). In contrast to mice, genes involved in intramyocellular beta-alanine oxidation were not upregulated, suggesting that this pathway mainly takes place in central organs and to a much smaller extent inside muscle cells. Whether beta-alanine can also be synthesized inside myocytes remains unclear and warrants further investigation. To strengthen and confirm these data, protein expression levels could be measured by Western Blot analysis. Therefore, these results are currently defined as preliminary.

REFERENCES

Baguet, A., Bourgois, J., Vanhee, L., Achten, E., & Derave, W. (2010). Important role of muscle carnosine in rowing performance. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *109*(4), 1096–101. doi:10.1152/japplphysiol.00141.2010

Blancquaert, L., Baba, S., Kwiatkowski, S., Stautemas, J., Stegen, S., Barbaresi, S., … Everaert, I. (2016). Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta-alanine transamination. *The Journal of Physiology*, *594*(17), 4849–63. doi:10.1113/JP272050.

Everaert, I., De Naeyer, H., Taes, Y., & Derave, W. (2013). Gene expression of carnosine-related enzymes and transporters in skeletal muscle. *European Journal of Applied Physiology*, *113*(5), 1169–79. doi:10.1007/s00421-012-2540-4

Harris, R. C., Tallon, M. J., Dunnett, M., Boobis, L., Coakley, J., Kim, H. J., … Wise, J. a. (2006). The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino Acids*, *30*(3), 279–89. doi:10.1007/s00726-006-0299-9

Hill, C. A., Harris, R. C., Kim, H. J., Harris, B. D., Sale, C., Boobis, L. H., … Wise, J. A. (2007). Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino Acids*, *32*(2), 225–33. doi:10.1007/s00726-006-0364-4

Lenney, J. F., Peppers, S. C., Kucera-orallo, C. M., & George, R. P. (1985). Characterization of human tissue carnosinase. *Biochemical Journal*, *228*, 653–660.

Romero, S. A., Hocker, A. D., Mangum, J. E., Luttrell, M. J., Turnbull, D. W., Struck, A. J., … Halliwill, J. R. (2016). Evidence of a broad histamine footprint on the human exercise transcriptome. *J Physiol*, (541), In press. doi:10.1113/JP272177

Stegen, S., Blancquaert, L., Everaert, I., Bex, T., Taes, Y., Calders, P., … Derave, W. (2013). Meal and beta-alanine coingestion enhances muscle carnosine loading. *Medicine and Science in Sports and Exercise*, *45*(8), 1478–85. doi:10.1249/MSS.0b013e31828ab073

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), 0034.1–0034.11.

III

General Discussion

1. Carnosine homeostatic set point

Carnosine is an intramuscular dipeptide, characterized by a high homeostatic set point. Based on the concept of homeostasis, it is hypothesized that muscle carnosine concentrations are kept within certain limits by a complex interplay of regulatory mechanisms. This thesis contributed to a better understanding of the muscle carnosine homeostatic regulation. Below, we will discuss the homeostatic set point and normal range that can be derived from our studies and the determinants that are further elucidated.

1.1. Homeostatic set point and normal range

Based on a database of proton MRS carnosine measurements in calf muscles that were collected over the past years in our lab, we generally presume that the set point amounts to 4.11 mM in soleus muscle and 6.96 mM in gastrocnemius muscle. In both study 2 (histidine supplementation) and study 3 (vegetarian intervention) of this thesis, we measured the soleus and gastrocnemius carnosine content of 30 and 40 subjects, respectively. By adding these new measurements to our database, we can constantly enlarge this database, thereby providing an adapted set point based on a bigger amount of measurements . Taking into account the new values provided by the studies included in this thesis, we can now state that the **carnosine set point amounts to 4.09 mM in soleus and 6.79mM in gastrocnemius**, which closely resembles the previous determined set points.

The carnosine set point in the specific populations of studies 2 and 3 at the start of the interventions amounts to 4.46mM and 7.26mM in study 2 and 3.65mM and 5.18mM in study 3 for soleus and gastrocnemius, respectively. The interindividual variation is 25.4% and 26.0% for study 2 and 24.8% and 27.8% for study 3 in soleus and gastrocnemius, respectively, which closely resembles the previous reported interindivual variation coefficients (Derave *et al.*, 2010). The fact that the set points in study 2 are higher than the ones in study 3 can most likely be explained by the fact that study 2 included both males and females, while study 3 only recruited females. It is generally assumed that the carnosine set point in men is approximately 20-25% higher compared to the set point in women (Mannion *et al.*, 1995). By separating the measurements of males and females of

our complete database (n = 387, including the measurements of this thesis), different Gaussion distributions can be derived (Fig 1). Indeed, both in soleus and gastrocnemius, the carnosine set point is 22% and 28% higher in men compared to women, respectively. Based on these data, we can confirm that **sex is indeed a determinants of the muscle carnosine set point**.

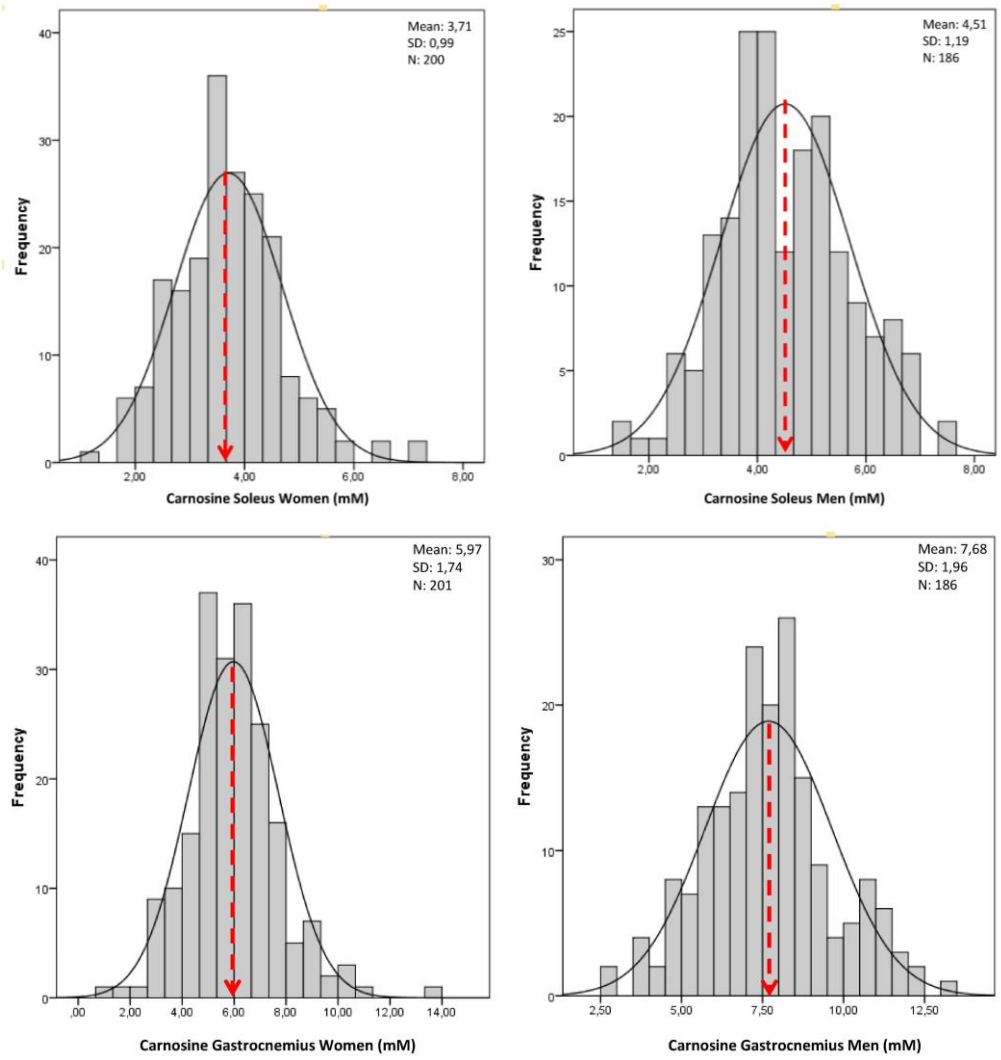

Figure 1: Gaussian distribution of the carnosine content in both soleus and gastrocnemius muscles in males and females. Data are compiled from the database of carnosine measurements from our lab, including the measurements used in the studies of this thesis

Repeated measurements of muscle carnosine levels over time in the same persons demonstrated a **low intra-individual variation** and thus a high stability of the carnosine set point. For soleus and gastrocnemius muscle, a previous study determined variation coefficients over a 3 month period and concluded the variation was 9% and 15%, respectively (Baguet *et al.*, 2009). Taking into account the methodological variation of the

MRS, the biological variation of muscle carnosine content over a 3 month period is as low as ~6% (Baguet *et al.*, 2009). In both study 2 and 3, three carnosine measurements were performed over time. This allows us to further elucidate whether carnosine is indeed a stable metabolite both on the short (23 days in study 2) and on the long term (6 months in study 3), compared to the 3 month period in which the stability is already demonstrated. In study 2, the intervention period lasted 23 days with an intermediate measurement at day 12. In this study, the carnosine content of the histidine-supplemented group was not affected by the intervention and it displayed a variation coefficient of 10.9% in soleus and 15.0% in gastrocnemius (without taking into account the variation of the MRS), which closely leans to the variation coefficients calculated by Baguet et al. (2009). Moreover, carnosine content was also determined by HPLC in a biopsy of the vastus lateralis muscle taking before and after the intervention period, demonstrating a variation coefficient of 15.5%. Thus, in another muscle and using another technique to determine carnosine content, a similar variation coefficient was found. In study 3, measurements were taken at 3 and 6 months following baseline measurements and included a control group who did not undergo any intervention. In these subjects, variation coefficients were 9.3% and 12.6% for soleus and gastrocnemius respectively, demonstrating that the carnosine set point is indeed stable over a longer period, which supports the theory that the carnosine set point and thus homeostasis is specifically regulated over time and that this stability extends to at least 6 months (Table 1).

Table 1. Coefficients of variation (CV) of the repeated measurements of carnosine, creatine and carnitine in the control group or intervention group with no effect. VL: vastus lateralis

	CV (%)					
	Study 2			Study 3		
	SOLEUS	GASTR	VL	SOL	GASTR	VL
Carnosine	10.9	15.0	15.5	9.3	12.6	
Creatine	No values available					7.8
Carnitine						7.0

In study 3, we did not only focus on carnosine homeostasis, but also measured 2 other carninutrients, namely creatine and carnitine, of which it can be hypothesized that their

intramyocellular content is also under homeostatic control. For these metabolites, we can only calculate the variation coefficient over 3 months since vastus lateralis biopsies at 6 months are lacking in this study. For total creatine and total carnitine, variation coefficient is 7.8% and 7.0% respectively, which is in the same range as the coefficients calculated for carnosine, thus suggesting that these carninutients are indeed subject to homeostatic regulation. However, for carnitine, reduced levels were found at 3 months and seemed to be restored by 6 months (based on results of plasma metabolites), which is suggested to reflect seasonal variations. Thus, for carnitine, it might be suggested that homeostasis is influenced by seasonal variations, which is not the case for carnosine or creatine, indicating that homeostatic regulation of the latter two is stronger than the regulation of carnitine. Since no effects of long-tem vegetarianism was found on carnitine homeostasis, it can be concluded that seasonal variations have a bigger impact on carnitine homeostasis compared to a vegetarian diet, which is practically free of carnitine.

1.2. Updated view on determinants of muscle carnosine set point

In contrast to the low intra-individual variability, there is a large inter-individual variation in the carnosine set point in human skeletal muscle. The described determinants for the muscle carnosine set point are age, gender, diet and muscle fiber type composition (Boldyrev *et al.*, 2013). Based on the measurements in our database, we could already confirm that gender is indeed a determinant of the baseline muscle carnosine set point, with a higher carnosine set point in men compared to women.

As mentioned, HCDs are present in meat and fish and omnivores thus have a daily dietary ingestion of these metabolites. It is already suggested that normal variations in the **dietary HCD** intake are not greatly affecting muscle carnosine homeostatic set point, but cross-sectional data on long-term vegetarians suggest that they have a somewhat lower muscle carnosine set point compared to omnivorous subjects (Harris *et al.*, 2007; Everaert *et al.*, 2011). This thesis contains the first longitudinal evidence demonstrating that muscle carnosine homeostasis is not affected by a 6-month vegetarian diet in previous omnivorous women (study 3), thus **nullifying the diet as an important determinant** of the muscle carnosine set point. An updated view of the determinants of baseline muscle carnosine is depicted in figure 2 (adapted from Derave et al. (2010)).

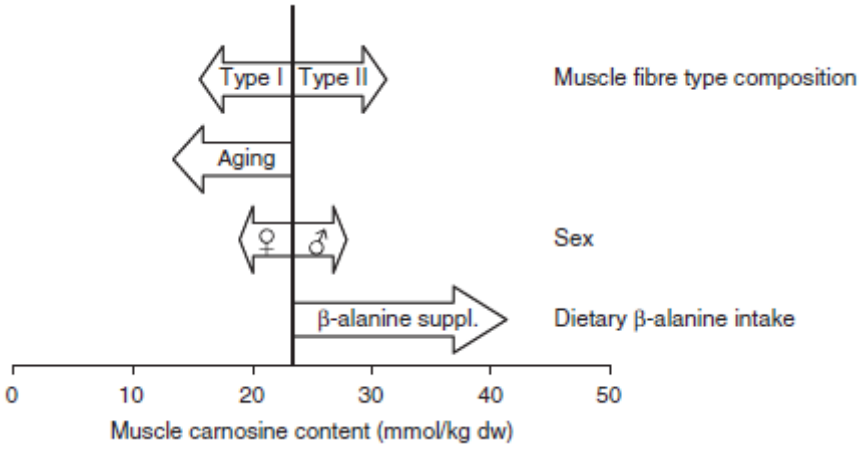

Figure 2: updated view on the determinants of baseline muscle carnosine content. Based on the results of study 3, it can now be stated that long-term vegetarianism is no longer a determinant.

Based on the vegetarian data, it can be concluded that the maintenance of muscle carnosine homeostasis does not depend on the nutritional supply of beta-alanine, suggesting that **endogenous beta-alanine synthesis** can entirely compensate for this absence. Next to uracil degradation in liver, which is a known pathway to synthesize beta-alanine, other possible beta-alanine synthesizing pathways include aspartate decarboxylation by GADL1 (Liu *et al.*, 2012*a*) or conversion of malonate semi-aldehyde by aminotransferase GABA-T and AGXT2. Both GADL1 and GABA-T are shown to be expressed in myocytes (study 4), while the expression of AGXT2 could not yet be demonstrated. Moreover, study 1 demonstrated that, upon beta-alanine supplementation, the transaminases regulate beta-alanine availability in the circulation by their high expression in liver and kidney. The beta-alanine synthesizing capacity of the transaminating pathways was not elucidated in this thesis and thus warrants further investigation.

In summary, the studies included in this thesis contribute to a better understanding of the baseline muscle carnosine homeostasis. We confirm that the carnosine set point is stable over a 6-month period (low intra-individual variability) and that men have a higher baseline set point compared to women (high inter-individual variability). A vegetarian diet is not a determinant of baseline muscle carnosine concentration, suggesting that endogenous beta-alanine synthesis is an important pathway and needs to be further investigated.

2. Carnosine loading protocol

Next to a better understanding of the regulation of baseline muscle carnosine homeostasis, this thesis further unraveled the carnosine loading process by beta-alanine supplementation. As described in the introduction, carnosine loading by beta-alanine supplementation is a rather inefficient process since only 2-3% of the ingested amount was shown to be incorporated into muscle carnosine. Meal co-ingestion was the first determinant of carnosine loading to be discovered (Stegen *et al.*, 2013*b*), as higher muscle carnosine loading was found when combining meal and beta-alanine intake, compared to taking beta-alanine tablets in between meals. Furthermore, Bex et al. (2014, 2015) unraveled that muscle carnosine loading efficiency is positively affected by an acute response of exercise training, with a possible additive effect of chronically trained muscles. This thesis further explored the low efficiency of carnosine loading by focusing on the metabolism of both precursor amino acids. The quota of **beta-alanine transamination** in the unknown metabolic fate of ingested beta-alanine was elucidated in **study 1**, while focus was shifted on **histidine** and its apparent unlimited availability for synthesis in **study 2**. With these studies, we aimed to enhance our knowledge about the carnosine metabolism on the one hand and tried to search for additional strategies to improve beta-alanine supplementation efficiency on the other hand.

2.1. Muscle carnosine homeostasis vs plasma beta-alanine homeostasis

Because beta-alanine supplementation disturbs plasma beta-alanine homeostasis and subsequently evokes an increase in muscle carnosine levels, the latter can be seen as an attempt to restore plasma beta-alanine levels. Therefore, it can be hypothesized that plasma beta-alanine homeostasis is more important and thus under a more strict homeostatic regulation than muscle carnosine homeostasis. As mentioned in the introduction, an enhanced stimulation of transsarcolemmal beta-alanine uptake by TauT and intramyocellular carnosine synthesis by CARNS upon elevated plasma beta-alanine levels in mice further supports this notion. These findings were now confirmed in humans (study 4) and an additional stimulation of beta-alanine transporter PAT1 was demonstrated, further confirming the transcriptional regulation aiming to enhance transsarcolemmal beta-alanine uptake following oral beta-alanine supplementation.

Upon beta-alanine supplementation, both CARNS and GABA-T mRNA expression was increased in mice skeletal muscle, while this could not be confirmed for GABA-T in human muscles. Based on these data, it is difficult to identify whether increased beta-alanine levels are primarily routed toward carnosine (CARNS) or toward oxidation (GABA-T and possibly also AGXT2). The results following orchidectomy in mice, a condition of presumed reduced sarcoplasmic beta-alanine availability, suggest that priority seems to be given to carnosine synthesis, as CARNS is strongly upregulated, whereas GABA-T was downregulated (Everaert *et al.*, 2013*a*). This observation led to **the 'old' working hypothesis that the priority role of beta-alanine is to serve as the precursor of carnosine synthesis**, and that the role of beta-alanine as a fuel is only used in conditions of excess availability. By displaying the beta-alanine and carnosine homeostasis in skeletal muscle as two communicating containers, this leads to the following graphical overview of the working hypothesis based on the results of Everaert et al. (2013*a*) (Fig 3).

Thus, as displayed in figure 3, this hypothesis assumes that, under normal conditions, some endogenous produced beta-alanine or beta-alanine from our diet can enter our muscle cells (depicted as the beta-alanine container) by TauT or PAT1. The availability of beta-alanine suffices to maintain the carnosine levels by the carnosine synthase enzyme. When beta-alanine is chronically supplemented, TauT and PAT1 are stimulated (bigger entrance for container), leading to an increased beta-alanine availiability inside myocytes. As a consequence, CARNS (and possibly GABA-T) is stimulated, and it is hypothesized that beta-alanine is primarily routed towards carnosine synthesis, while the rest of the remaining beta-alanine is transformed into malonate semi-aldehyde by GABA-T (and AGXT2) which can further be oxidized in the citric acid cycle.

Based on our results in study 1, this hypothesis does not longer hold since we demonstrated that, when low amounts of beta-alanine are supplemented (0.1% w/v in mice), plasma beta-alanine and muscle carnosine levels are not affected. However, when combining this dose with AOA, an inhibitor of both GABA-T and AGXT2, plasma beta-alanine levels are significantly increased and subsequently enhance muscle carnosine content. Based on these data, we can conclude that beta-alanine transamination is the main pathway of excess beta-alanine, and carnosine loading thus only appears when these enzymes are blocked or saturated. Thus, while Everaert et al. (2013*a*) hypothesized

that beta-alanine transaminases are activated when carnosine synthesis is saturated, we now prove it is the other way around; **net carnosine synthesis only occurs when beta-alanine transaminases are saturated** and is, next to beta-alanine transamination, thus a second pathway to restore circulating beta-alanine levels (Fig 3). These results confirm our previous hypothesis, namely that plasma beta-alanine homeostasis is more strictly regulated than tissue carnosine homeostasis. Nevertheless, these data also demonstrated that the body has a first line of defence against disturbed circulating beta-alanine levels (i.e. transamination of beta-alanine, subsequently used for energy delivery) and thus also severely endeavors to maintain tissue carnosine homeostasis. It can be concluded that **priority is given to plasma beta-alanine homeostasis**, but **tissue carnosine homeostasis is also greatly attempted to maintain constant**.

The mRNA expression profile of carnosine-related enzymes and transporters in different mice tissues confirmed a high expression of these enzymes in liver and kidney (and GABA-T also in brain tissue) and a low expression in muscles (study 1). Muscle GABA-T mRNA expression was not upregulated following beta-alanine supplementation in humans. Together, these data suggest that a whole body regulation is involved in the circulating beta-alanine and tissue carnosine homeostasis and regulation is not limited to muscle level. Thus, in contrast to formerly believed, the containers of figure 3 can no longer be seen as muscle cells but must be seen as an interaction between plasma and tissues (left barrel is plasma beta-alanine, right barrel is tissue carnosine).

The priority to maintain plasma beta-alanine levels is in accordance with the notion that enhanced plasma beta-alanine levels cause **parasthesia** (transient and unpleasant flushing sensations on the skin) (Harris *et al.*, 2006). These neural side-effects are caused by the high peak plasma beta-alanine concentrations upon ingestion of a pure beta-alanine supplement (>10mg/kg body weight) and has been shown to be mediated by MrgprD receptor expressed in cutaneous sensory neurons (Liu *et al.*, 2012*b*). In contrast, enhanced muscle carnosine levels are beneficial towards performance and do not induce any negative side-effects. In this respect, disturbing tissue carnosine homeostasis is a logic mechanism to abolish plasma beta-alanine and prevent parasthesia.

To summarize, we now generated a better understanding of the muscle carnosine metabolism and proved that tissue carnosine is a strict homeostasis in which beta-alanine transaminases are involved to maintain the carnosine homeostasis as long as possible during beta-alanine supplementation. Only by saturating this pathway, carnosine loading eventually appears. This also explains the long and intensive beta-alanine supplementation protocol and the low incorporation efficiency of beta-alanine into muscle carnosine, which is further discussed below.

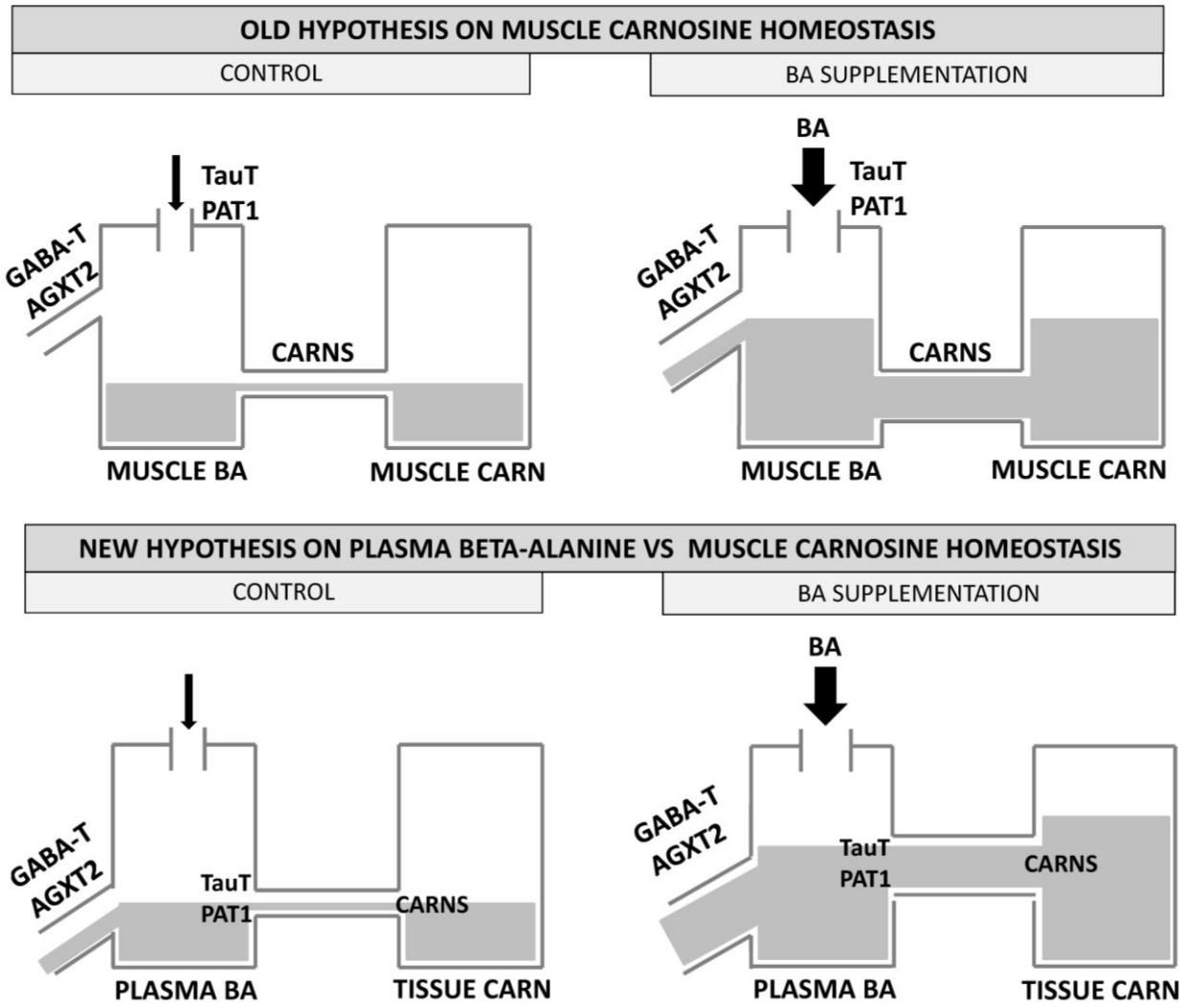

Figure 3: graphical overview of the old hypothesis on regulation of muscle carnosine levels and the new hypothesis on plasma beta-alanine vs muscle carnosine homeostatic regulation, based on study 1 of this thesis.

Next to the presence of transaminases to remove excess beta-alanine, a second argument demonstrating that tissue carnosine set point is kept as stable as possible is provided in the 6-month vegetarian intervention, which did not affect muscle carnosine levels, thus

suggesting that the endogenous beta-alanine synthesis can entirely compensate for the absence of dietary beta-alanine. The expression of GADL1 found in human muscle indicate that there indeed might be an intramyocellular pathway to synthesize beta-alanine, although it is more likely that this enzyme is clearly expressed and/or active in other organs, such as the liver (where uracil degradation takes place) and thus beta-alanine synthesis is, alike beta-alanine degradation, regulated more centrally.

2.2. L-histidine homeostasis

Until now, we mainly focused on circulating beta-alanine and tissue carnosine homeostasis. However, as mentioned, L-histidine is the other precursor amino acid for carnosine synthesis, although not much attention is currently paid to this amino acid in literature. It is generally believed that in humans, L-histidine is sufficiently available and thus not rate-limiting in the carnosine synthesis process. This is in contrast with some animal species, in which histidine supplementation has been shown to increase muscle carnosine levels (Tamaki *et al.*, 1977; Park *et al.*, 2013). The high availability of human L-histidine compared to beta-alanine is demonstrated by fasted plasma L-histidine concentrations in the range of 40-60µM (in contrast to 2-5µM for beta-alanine) and intramyocellular L-histidine concentrations in the range of 0.2mmol/kg wet weight (in contrast to 40-100µmol/kg wet weight for beta-alanine). However, the histidine levels upon chronic beta-alanine supplementation were until now never monitored and we demonstrated for the first time that **L-histidine is depleted by chronic beta-alanine supplementation in both plasma and muscle**, indicating that body L-histidine homeostasis is disturbed. These results suggest that endogenous L-histidine synthesis can not compensate for reduced L-histidine levels. Based on these findings, we can conclude that beta-alanine homeostasis is considered more important than L-histidine homeostasis, since beta-alanine is upon supplementation partly oxidized and additionally used for carnosine synthesis, although this depletes histidine stores.

It can be hypothesized that **disturbed L-histidine homeostasis may have some implications**. Next to beta-alanine transamination, reduced L-histidine levels might be a second explanation for the low efficiency of chronic beta-alanine supplementation toward carnosine loading. This could not yet be demonstrated in study 2 of this thesis; co-supplementation of beta-alanine and L-histidine did not significantly enhance the amount

of carnosine loading. However, we found a more modest decline in the amount of carnosine loading during the second part of supplementation (D12-D23) in the co-supplemented group compared to the beta-alanine supplementation group, suggesting that the **depletion of plasma and muscle histidine could become more problematic in longer duration studies** with higher amounts of supplemented beta-alanine.

Indeed, figure 4 gives a graphical overview of different studies that chronically supplemented beta-alanine and measured carnosine levels at different timepoints, thereby making it possible to calculate beta-alanine efficiency throughout a chronic supplementation period. For example, the study of Hill et al. (2007) measured carnosine loading in the vastus lateralis after 4 and 10 weeks of beta-alanine supplementation and reported an increase in carnosine levels of 58.8% at 4 weeks and 80.1% at 10 weeks (compared to baseline), demonstrating a lower amount of loading in the second part of supplementation. When calculating the incorporation efficiency for beta-alanine, this was 5.0% in the first 4 weeks vs 1.22% in the subsequent 6 weeks (Fig 4), highlighting that the efficiency of beta-alanine supplemention is already low at the start and is even fastly decreasing during chronic supplementation. Similarly, the study of Stellingwerff et al. (2012*a*) compared two loading protocols; a high-low protocol (3.2g/d during 4 weeks followed by 1.6g/d during 4 weeks) and a low-low loading protocol (1.6g/d during 8 weeks) and measured carnosine loading at 2, 4 and 8 weeks. Based on these values, efficiency of carnosine loading can be measured for the first two weeks, the subsequent two weeks and the last 4 weeks, separately. As depicted in figure 4, efficiency is stable after 2 and 4 weeks and equal for the two dosing protocols. The low-low group ingested half of the amount of beta-alanine (44.8g) at 4 weeks and showed a carnosine increase of 19% in tibialis anterior muscle, while the high-low group (ingested 89.6g) increased by 36% after 4 weeks (thus, both beta-alanine intake and carnosine loading are doubled, resulting in the same efficiency). Similar to the results of Hill et al. (2007), the efficiency is decreasing after 4 weeks of supplementation in both dosing protocols, and this decline is steeper for the high-low group compared to the low-low group. These results indicate that the efficiency of beta-alanine supplementation seems to decrease after 4 weeks for the supplementation protocols used in the above described studies. Moreover, the higher the amount of carnosine loading (and thus histidine depletion), the greater this decline.

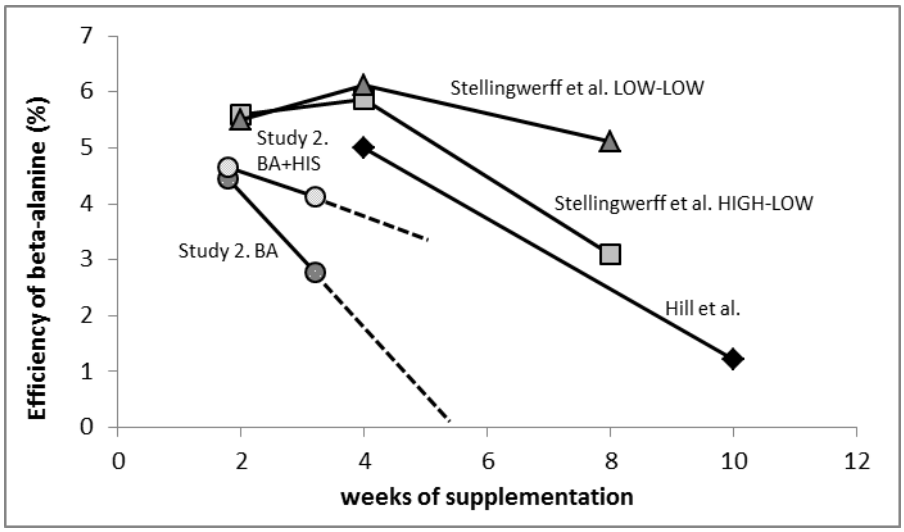

Figure 4: Efficiency of chronically supplemented beta-alanine, calculated in different supplementation studies and study 2 of this thesis. Efficiency is calculated by dividing the molar carnosine increase by the total molar intake of beta-alanine.

By calculating the efficiency for study 2, we can compare these values and the course of the efficiency during chronic supplementation. In study 2, 6g of beta-alanine was ingested daily, which is higher than the supplemented amounts of Hill et al. (2007) and Stellingwerff et al. (2012*a*). Figure 4 shows that efficiency was already decreasing after 12 days of supplementation, and this decline was more modest in the co-supplemented group compared to the beta-alanine group, suggesting that histidine depletion might affect the amount of carnosine loading. However, we were not able to find significant differences in the amount of loading due to the short supplementation period, but it can be hypothesized that a significant difference in loading would appear when supplementation was continued (as indicated by the dashed lines on figure 4).

Furthermore, L-histidine is, in contrast to beta-alanine, a proteinogenic amino acid and thus necessary for muscle synthesis. Therefore, it could be hypothesized that L-histidine would be even more depleted in **strength training athletes** and thus can more rapidly become rate-limiting for carnosine synthesis. Moreover, subjects characterized by a lower intake of proteins (which is the main source of dietary histidine), such as **vegetarians and elderly** are other populations in which histidine depletion may occur to a higher extent. Whether L-histidine indeed becomes rate-limiting when beta-alanine is supplemented in these populations remains to be investigated. However, our results suggest that L-histidine levels should be monitored when beta-alanine is chronically ingested in these subjects.

To summarize, body histidine stores are depleted by chronic beta-alanine supplementation. However, this does not seem to affect muscle carnosine loading on the short term, as was tested in study 2. Nevertheless, it might affect the loading efficiency in longer supplementation protocols and thus co-supplementation of beta-alanine and L-histidine warrants further research.

A possible exercise-related metabolic pathway in which histidine is involved, is **histamine synthesis through decarboxylation of L-histidine** by HDC. We could not measure HDC expression in human skeletal muscle, but its expression in muscle has been demonstrated by other recent investigations (Romero *et al.*, 2016). It is suggested that histamine, which has beneficial exercise-related roles, might derive from muscle histidine. In this regard, carnosine is often seen as a histidine pool (Greene *et al.*, 1984). In case of decreased plasma histidine levels, it can be hypothesized that HDC is downregulated and carnosine degradation is increased (as was found by the higher expression of CNDP2 in study of Everaert et al. (2013*a*)), while the opposite might appear in case of increased plasma histidine levels (Fig 4). However, until now, it remains to be investigated if and how carnosine and HDC contribute to the maintenance of histidine homeostasis.

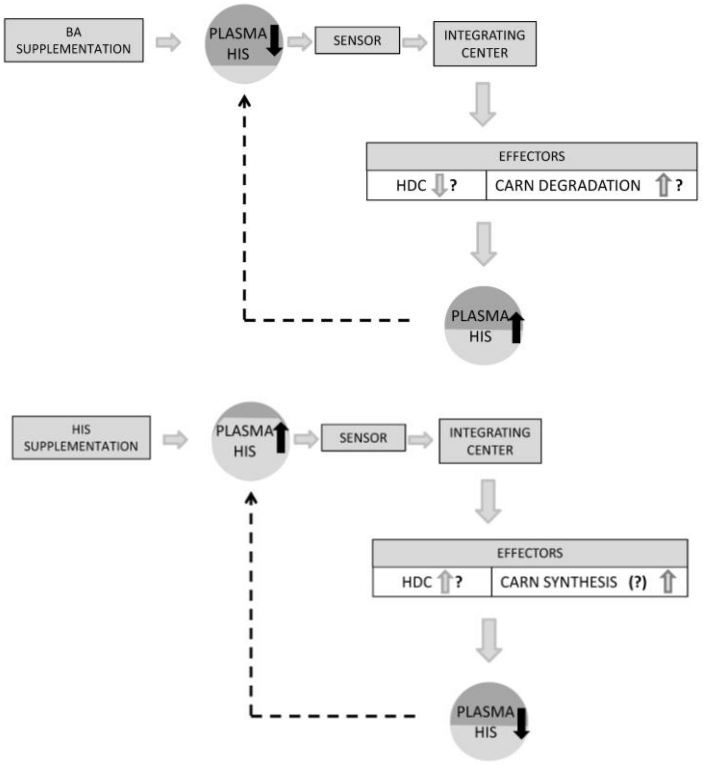

Figure 5: Possible hypothesis on the interaction of carnosine metabolism with the homeostatic regulation of histidine levels

2.3. Updated view on efficiency of beta-alanine supplementation

Based on our studies, we can conclude that depleted histidine levels following chronic beta-alanine supplementation might negatively affect carnosine loading, and we demonstrated that beta-alanine transaminases are indeed partially responsible for the low efficiency of beta-alanine supplementation.

By concurrent administration of beta-alanine and beta-alanine transaminases inhibitors in mice, we demonstrated that higher amounts of loading are evoked when both GABA-T and AGXT2 are blocked. Based on these data, we can now partially explain the low loading efficiency of beta-alanine supplementation. As this study was performed in mice, it is difficult to estimate the contribution of these enzymes to the total unknown metabolic fate of ingested beta-alanine in humans. Moreover, the effect of inhibiting GABA-T and AGXT2 on the mice muscle carnosine loading differed greatly between the different muscles that were investigated (small effects for gastrocnemius and tibialis anterior, big effects for soleus and heart). Further research on the contribution of beta-alanine transaminating enzymes in humans and other possible determinants of carnosine loading is needed.

As described above, the highest effects of beta-alanine supplementation and concurrent beta-alanine transaminase inhibition were found in soleus and heart (study 1). Moreover, **serum beta-alanine was positively correlated with the HCD levels** in these tissues. Fasted plasma beta-alanine levels of humans supplemented with beta-alanine (with or without concurrent histidine supplementation) were also measured in study 2 of this thesis. Interestingly, change in fasted plasma beta-alanine levels after 23 days of supplementation demonstrated a significant correlation with the change in muscle carnosine levels in soleus ($r = 0.591$, $p = 0.01$) and gastrocnemius ($r = 0.442$, $p = 0.067$) muscle (Fig 6). Moreover, a similar correlation was already present after 12 days of supplementation, although this was only significant in gastrocnemius ($r = 0.503$, $p = 0.04$) and not in soleus ($r = 0.291$, $p > 0.05$). Thus, similar to the results found in mice (study 1), **fasted plasma beta-alanine levels seem a good predictor for the amount of muscle carnosine loading**. This is in agreement with the above described hypothesis that plasma beta-alanine homeostasis is strictly regulated. When these levels can not be kept within homeostatic limits (e.g.saturation of transaminases), beta-alanine is transported inside

muscle cells and carnosine synthesis thus is a consequence of the disturbed plasma beta-alanine homeostasis. Taken together, the data from different studies included in this thesis indicate that the change in fasted plasma beta-alanine levels following chronic beta-alanine supplementation can be seen as an indication for the amount of muscle carnosine loading.

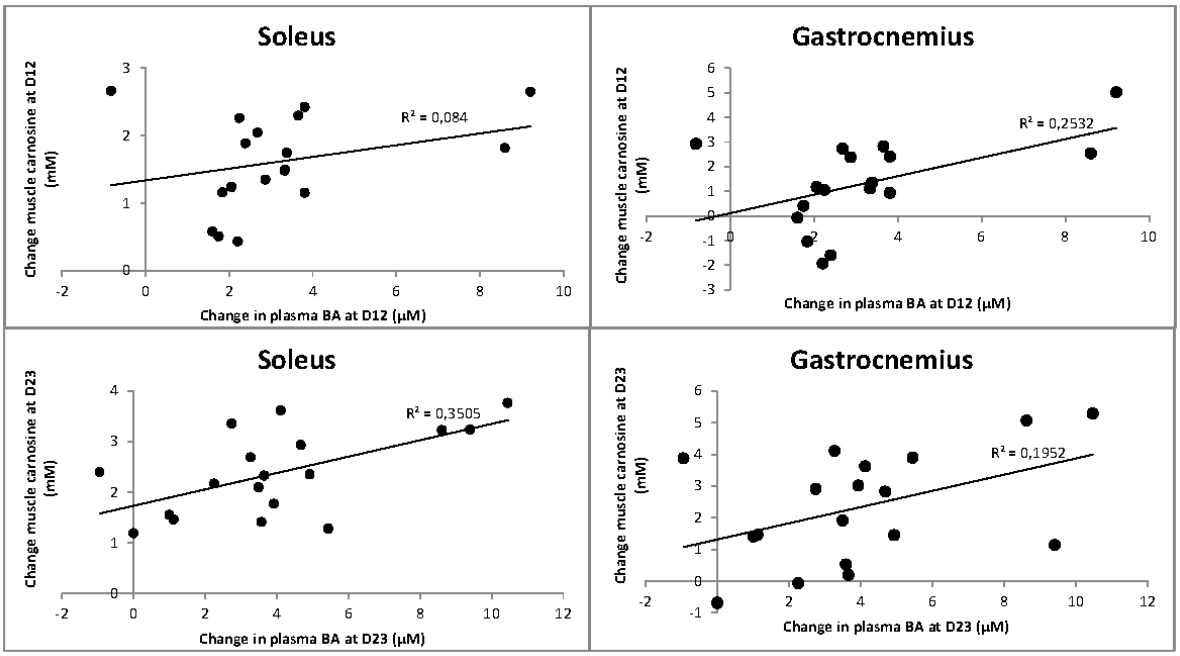

Figure 6: Correlation between the change in plasma beta-alanine levels and the change in muscle carnosine levels for soleus and gastrocnemius muscle of the beta-alanine supplemented subjects in study 2

This finding might have some important implications. Fasted plasma beta-alanine levels are collected 8-10 hours after the last beta-alanine intake. We demonstrated that these fasted beta-alanine levels are correlated with muscle carnosine increase both after 12 and 23 days of supplementation. Thus, at these timepoints, fasted plasma beta-alanine is a predictor for the responsiveness of a subject to beta-alanine supplementation. This is probably a consequence of the GABA-T and/or AGXT2 activity. The higher this activity, the lower plasma beta-alanine levels and thus the lower muscle carnosine loading. In that case, it could be hypothesized that a similar correlation already occurs after one single day of beta-alanine supplementation, e.g. after supplementation of 6.4g of beta-alanine for one day, fasted plasma beta-alanine levels could be measured on the next morning and correlated with carnosine increase after chronic supplementation. If this correlation holds, this would mean that the change in plasma beta-alanine levels after one single day

of supplementation is a predictor for the degree of carnosine loading after chronic beta-alanine supplementation. Thus, disturbed plasma beta-alanine homeostasis after one day of supplementation could then be a indication that this subject is a high-responder to beta-alanine supplementation.

As demonstrated in study 1, plasma beta-alanine homeostasis is mainly regulated by renal and hepatic beta-alanine transamination by GABA-T and AGXT2. In this study, we could confirm that beta-alanine is a suitable substrate for both GABA-T and AGXT2. These enzymes are thus responsible for the removal of beta-alanine in plasma. Furthermore, beta-aminoisobutyric acid (BAIBA) is another suitable substrate for AGXT2, as was also demonstrated in study 1. Interestingly, few recent studies demonstrated that certain **SNPs (single nucleotide polymorphism) are associated with reduced activity of AGXT2** in humans (Suhre *et al.*, 2011; Yoshino *et al.*, 2014). The frequency of these SNPs is population-dependent and is more frequently present in Asian populations (up to 33%) compared to Caucasian populations (less than 1%) (Auton *et al.*, 2015). In these subjects, BAIBA urinary excretion was significantly enhanced (Yoshino *et al.*, 2014), suggesting that less BAIBA is transaminated by AGXT2, thus leading to a higher excretion. Based on these findings, it could be hypothesized that these subjects also have reduced transamination of circulating beta-alanine, resulting in enhanced plasma beta-alanine levels upon beta-alanine supplementation and consequently a higher degree of carnosine loading. However, until now, the effect of beta-alanine supplementation in these 'AGXT2-deficient' subjects is not yet investigated and might be an interesting study to investigate the contribution of AGXT2 to the plasma beta-alanine homeostasis in humans.

To summarize, fasted plasma beta-alanine levels are a predictor for the degree of muscle carnosine loading. In other words, subjects who fail to keep circulating beta-alanine between homeostatic limits, will have a higher incorporation of beta-alanine into muscle carnosine and thus a higher degree of muscle carnosine homeostasis failure. These subjects are the high-responders to beta-alanine supplementation.

3. Limitations

In this thesis, new insights in the regulation of the carnosine homeostasis are discovered. However, some limitations of the performed studies are highlighted below.

- Throughout the different studies of this thesis, quantitative PCR is used to measure the mRNA expression of the carnosine-related enzymes and transporters. However, it is known that mRNA expression is not always in agreement with the amount of mRNA that is effectively translated into protein. Discordance between gene and protein expression levels is previously described for different genes and might be caused by different factors, such as post-transcriptional splicing and translational regulation. Thus, true biological differences might exist between mRNA and protein levels, and gene expression measurements should therefore be interpreted with caution as they only give an estimate of the correspondent protein expression and activity. To truly measure protein expression, Western blot analysis should be performed, and activity assays are considered as the gold standard to meaure protein function, as was done for GABA-T and AGXT2 in study 1.

- The performed studies have a relatively small sample size, thereby lowering the power of the studies. In study 2 and 3, the interventions were quite intensive, making it inconvenient to include more subjects. In the context of the reduction principle for the use of laboratory animals, we also kept the groups in study 1 quite small. For some parameters however, it might be more straightforward to find significances in a larger sample size (e.g. taurine in study 2).

- Thirdly, the moment on which the biopsy was taken can have a great influence on the gene expression results. Since we only take one biopsy after a chronic supplementation period in study 4, the results only give a snapshot of the gene expression profiles on that moment. It is possible however that gene expression is more acutely influenced and affected by a stimulus and is already returning to baseline after a chronic intervention.

- The contribution of beta-alanine transaminases in the carnosine homeostasis in mice was demonstrated by vigabatrin and AOA administration, which are inhibitors of GABA-T or both GABA-T and AGXT2, respectively. Based on our findings in mice, we are unable to determine the fraction of ingested beta-alanine in humans that is lost through

transamination. Moreover, our findings can not directly be applied to the human situation because of some reported side-effects of vigabatrin and the toxicity of AOA. Vigabatrin is sometimes used as a drug for people with epilepsy or refractory complex partial seizures, but it is not a first line treatment as it can cause drowsiness, dizziness and permanent vision loss (deterioration of sight from the edges of your field of vision). Furthermore, AOA inhibits all PLP-dependent enzymes and was already tested for the treatment of some diseases, leading to toxic symptoms such as drowsiness, ataxia, seizures, and psychotic behavior (Perry *et al.*, 1980). It remains to be established whether beta-alanine transaminases can be blocked by an alternative way which can also be applied in humans.

- Because all PLP-dependent enzymes are blocked by AOA administration, we can currently not state with certainty that no other unknown beta-alanine transaminase enzyme is involved in this process. Moreover, inhibitor administration was only tested in combination with beta-alanine administration. It could however be hypothesized that blockade of the transamination of endogenous beta-alanine can elicit muscle carnosine loading, thus making beta-alanine supplementation unnecessary.

4. Practical applications

Based on the results of this thesis, we can formulate the following practical applications:

- Beta-alanine is a popular ergogenic supplement among athletes. It was already demonstrated that meal co-ingestion and exercise training are determinants for the beta-alanine induced carnosine loading scheme. Therefore, athletes are adviced to take the beta-alanine supplement together with meals and or training sessions and to plan the supplementation period during a training period. Our results now demonstrate that a large amount of the ingested beta-alanine is **primarily routed toward energy provision** (by transamination of beta-alanine to malonate semi-aldehyde) and beta-alanine is only routed toward carnosine synthesis when **the transaminase pathway is saturated**. We demonstrated that short and intensive beta-alanine supplementation (6gr per day during 23 days) already evoked significant increases in muscle carnosine. Thus, similar amounts of carnosine loading can be obtained when maximizing the daily beta-alanine intake during a limited period of time instead of spreading smaller doses during a longer period, which might be more convenient for athletes preparing for a championship/competition.

- If beta-alanine is supplemented for a prolonged period of time, caution should be warranted to **body histidine levels**. Populations having a higher risk of incurring a histidine deficit, such as strength training athletes, growing children, vegetarians or elderly, should be cautious with beta-alanine supplementation on the long term.

- Creatine homeostasis was shown to be disturbed by a 6-month longitudinal vegetarian intervention in previous omnivorous subjects and could be restored by creatine supplementation (1g creatine monohydrate). Creatine plays a crucial bioenergetic role in tissues with high metabolic demand by re-synthesizing adenosine triphosphate (ATP) as a high-energy phosphate. Thus, **creatine is a suitable carninutrient for supplementation** in vegetarian athletes, especially those participating in resistance training and short-term, high-intensity exercise performances such as sprinting. Furthermore, creatine has emerged as a relevant dietary intervention able to partially offset frailty in the elderly and is also suggested to act as an antiglycemic agent (Gualano *et al.*, 2016; Pinto *et al.*, 2016). Therefore, creatine supplementation in specific clinical populations on a vegetarian diet might also have clinical relevance.

5. Future directions

The overall aim of this thesis was to provide a better insight in the homeostatic regulation of carnosine and thereby partly explaining the low efficiency of chronic oral beta-alanine supplementation. Although we provided new insights, some elements of the regulation of carnosine homeostasis remain unclear. Below, some **specific next steps** are described.

- We demonstrated that specific GABA-T inhibition by vigabatrin did not have an effect on the HCD content in any of the investigated tissues. Because we did not have an inhibitor specific for AGXT2, which does not act on GABA-T, the **contribution of GABA-T to the beta-alanine metabolism** needs further investigation. In order to be able to compare the implications of inhibiting only AGXT2 vs inhibiting both enzymes on the metabolism of beta-alanine in mice, AGXT2 knock-out mice are a suitable model. These mice are already successfully bred, but their muscle carnosine and anserine levels are until now never determined. If a low amount of beta-alanine supplementation (0.1% w/v) in these mice would directly enhance circulating beta-alanine and muscle carnosine levels, this would suggest that AGXT2 is the main enzyme that is responsible for beta-alanine transamination and GABA-T is only minimally involved. If no enhanced beta-alanine and carnosine levels would be present upon beta-alanine administration, this would be an indication that GABA-T also plays an important role in the transamination process. In this case, it can be suggested that the enzymes are both involved and they are able to compensate for the loss in activity of the other enzyme.

- The beneficial effects of administering transaminase inhibitors on the carnosine loading can not directly be transferred to humans because of the side-effects and toxicity of these substances. As an alternative**, a natural inhibitor** of these enzymes could be used. Lemon balm (Melissa Officinalis) is a natural herb and has already been shown to inhibit GABA-T activity both in vitro (Awad et al., 2007, 2009) and in vivo (Yoo et al., 2011). Alike vigabatrin, the inhibitory effects of lemon balm were shown to affect the GABA metabolism. Since lemon balm only inhibits GABA-T without affecting AGXT2 activity, it remains to be investigated whether beta-alanine metabolism will be affected by this substance. A natural inhibitor of AGXT2 is until now never demonstrated, but specific SNPs associated with decreased AGXT2 activity are described. Thus, administering lemon

balm to a small cohort of AGXT2 deficient patients would be a way to determine the contribution of both these enzymes to the carnosine homeostasis.

- Whether **histidine can become a rate-limiting factor for carnosine synthesis** needs further investigation in specific situations and populations such as long-term intensive beta-alanine supplementation, vegetarians, strength training athletes or elderly. If combined supplementation of beta-alanine and L-histidine would induce a higher amount of carnosine loading compared to beta-alanine supplementation alone, the limiting role of L-histidine would be demonstrated.

Besides these specific next steps that need to be further investigated on the short-term, there still are some **general gaps in the story of carnosine regulation**. Some suggestions and recommendations for future research tracks are defined below.

- Until now, apart from uracil degradation in the liver, very little is known about the **capacity of the body to synthesize beta-alanine**. Based on the results of this thesis, it can be hypothesized that this pathway might be of greater importance than previously thought. Indeed, van Kuilenburg and colleagues (2004) reported that plasma beta-alanine homeostasis is not disturbed in patients deficient in beta-ureidopropionase (last enzyme involved in endogenous beta-alanine synthesis by uracil degradation). Together with the results found in vegetarians (no exogenous beta-alanine), these data suggest that either other endogenous beta-alanine synthesizing pathways exist, or beta-alanine degradation is down regulated in these specific conditions. Future research is needed to elaborate whether, apart from uracil degradation in liver, other beta-alanine synthesis pathways exist and to what extent they contribute to the total endogenous beta-alanine availability. Possible pathways are aspartate decarboxylation by GADL1 and conversion of malonate semi-aldehyde by aminotransferases GABA-T and AGXT2. The mRNA expression of GADL1 found in human skeletal muscle suggests that this pathway indeed is involved, but the protein expression and enzyme activity remains to be investigated.

- Serum carnosinase (CN1), the enzyme responsible for the hydrolylis of carnosine, is in contrast to rodents, highly active in blood and therefore a negative regulator of carnosine content in humans. This surprisingly high carnosinase activity results in the absence of carnosine from the human blood, which causes the **'human carnosinase paradox'**. Up to

now, it is not yet clear why such an active enzyme evolves, when it degrades a molecule which has protective properties for a number of diseases and which promotes exercise performance capacity.

- **The link between the histamine and carnosine metabolism** remains until now unclear. Several functions are ascribed to histamine, of which the vasodilating effect on arterioles in (post-)exercise hyperaemia is a very interesting one toward exercise performance. Histamine might be stored in mast cells, or it can be formed by decarboxylation of muscle histidine. HDC is shown to be expressed in human skeletal muscle and is upregulated following exercise (Romero *et al.*, 2016). Therefore, carnosine metabolism may be closely related to histamine metabolism, which warrants further research.

- Due to the wide spectrum of bioactive properties of carnosine, its therapeutic potential has been tested in numerous diseases in which ischemic or oxidative stress are involved. Promising results have been obtained for several pathologies such as diabetes and its complications, ocular disease, aging and neurological disorders. In this dissertation, it was demonstrated for the first time that carnosine concentrations can be increased in the heart of rodents. As it was recently reported that carnosine plays an important role in detoxifying reactive aldehydes and promote functional recovery in the ischemic heart, it should be further investigated whether **increased carnosine levels can positively influence cardiac function** and resistance of the heart to ischemic injury. This would enhance the therapeutic potential of carnosine.

6. General conclusions

Take-home messages derived from this thesis:

- **Homeostasis of the HCDs in cardiac myocytes and skeletal muscle is dependent on the circulating availability of beta-alanine. In turn, homeostasis of circulating beta-alanine is, in the case of excess dietary beta-alanine intake, dependent on the degradation of beta-alanine in liver and kidney, which express GABA-T and AGXT2 as the main mammalian enzymes capable of metabolizing beta-alanine.** Plasma beta-alanine homeostasis is thus securely regulated by the human body. In first instance, enhanced circulating beta-alanine levels upon beta-alanine supplementation are routed towards oxidation. Secondly and only when this first pathway is saturated, beta-alanine is routed toward carnosine synthesis, resulting in disturbed muscle carnosine homeostasis. This partially explains the low incorporation efficiency of chronically ingested beta-alanine into muscle carnosine.

- **Beta-alanine is confirmed as the rate-limiting precursor in the muscle carnosine synthesis process** since histidine supplementation alone did not suffice to increase muscle carnosine content and adding histidine to the beta-alanine supplementation protocol did not improve short-term loading efficiency. Moreover, it is demonstrated for the first time that the **increase in fasted plasma beta-alanine levels following chronic beta-alanine supplementation is a determinant of the degree of muscle carnosine loading.**

- **Chronic beta-alanine supplementation reduces plasma and muscle histidine levels, which could be prevented by co-supplementing L-histidine alongside beta-alanine.** It remains to be determined whether the depletion of histidine levels by beta-alanine can compromise physiological processes such as carnosine loading of longer duration or protein synthesis in an anabolic state.

- **Creatine, but not carnosine and carnitine homeostasis, is disrupted by a 6-month vegetarian diet in omnivorous women**. Creatine homeostasis can be restored by concomitant creatine supplementation, suggesting that creatine is a suitable

carninutrient for supplementation in vegetarian athletes, especially those participating in resistance training and short-term, high-intensity exercise performances.

IV

References

Abe H (2000). Role of Histidine-Related Compounds as Intracellular Proton Buffering Constituents in Vertebrate Muscle. **65,** 757–765.

Altorf-van der Kuil W, Brink EJ, Boetje M, Siebelink E, Bijlsma S, Engberink MF, van 't Veer P, Tome D, Bakker SJL, van Baak MA & Geleijnse JM (2013). Identification of biomarkers for intake of protein from meat, dairy products and grains: a controlled dietary intervention study. *Br J Nutr* **110,** 810–822.

Amend J, Strumeyer D & Fisher H (1979). Effect of dietary histidine on tissue concentrations of histidine-containing dipeptides in adult cockerels. *J Nutr* **109,** 1779–1786.

Anderson CMH, Howard A, Walters JRF, Ganapathy V & Thwaites DT (2009). Taurine uptake across the human intestinal brush-border membrane is via two transporters: H+-coupled PAT1 (SLC36A1) and Na+- and Cl(-)-dependent TauT (SLC6A6). *J Physiol* **587,** 731–744.

Artioli GG, Gualano B, Smith A, Stout J & Lancha AH (2010). Role of beta-alanine supplementation on muscle carnosine and exercise performance. *Med Sci Sports Exerc* **42,** 1162–1173.

Auton A et al. (2015). A global reference for human genetic variation. *Nature* **526,** 68–74.

Awad R, Levac D, Cybulska P, Merali Z, Trudeau VL & Arnason JT (2007). Effects of traditionally used anxiolytic botanicals on enzymes of the gamma-aminobutyric acid (GABA) system. *Can J Physiol Pharmacol* **85,** 933–942.

Awad R, Muhammad A, Durst T, Trudeau VL & Arnason JT (2009). Bioassay-guided Fractionation of Lemon Balm (Melissa officinalis L .) using an In Vitro Measure of GABA Transaminase Activity. **1081,** 1075–1081.

Baba SP, Hoetker JD, Merchant M, Klein JB, Cai J, Barski O a, Conklin DJ & Bhatnagar A (2013). Role of aldose reductase in the metabolism and detoxification of carnosine-acrolein conjugates. *J Biol Chem* **288,** 28163–28179.

Baguet A, Bourgois J, Vanhee L, Achten E & Derave W (2010*a*). Important role of muscle carnosine in rowing performance. *J Appl Physiol* **109,** 1096–1101.

Baguet A, Everaert I, Achten E, Thomis M & Derave W (2012). The influence of sex, age and heritability on human skeletal muscle carnosine content. *Amino Acids* **43,** 13–20.

Baguet A, Everaert I, De Naeyer H, Reyngoudt H, Stegen S, Beeckman S, Achten E, Vanhee L, Volkaert A, Petrovic M, Taes Y & Derave W (2011). Effects of sprint training combined with vegetarian or mixed diet on muscle carnosine content and buffering capacity. *Eur J Appl Physiol* **111,** 2571–2580.

Baguet A, Koppo K, Pottier A & Derave W (2010*b*). Beta-alanine supplementation reduces acidosis but not oxygen uptake response during high-intensity cycling exercise. *Eur J Appl Physiol* **108,** 495–503.

Baguet A, Reyngoudt H, Pottier A, Everaert I, Callens S, Achten E & Derave W (2009). Carnosine loading and washout in human skeletal muscles. *J Appl Physiol* **106,** 837–842.

Bakardjiev A & Bauer K (1994). Transport of beta-alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture. *Eur J Biochem* **225,** 617–623.

Balsom PD, Söderlund K & Ekblom B (1994). Creatine in Humans with Special Reference to Creatine Supplementation. *Sport Med* **18,** 268–280.

Basiotis PP, Welsh SO, Cronin FJ, Kelsay JL & Mertz W (1987). Number of days of food intake records required to estimate individual and group nutrient intakes with defined confidence. *J Nutr* **117,** 1638–1641.

Bauer K & Schulz M (1994). Biosynthesis of carnosine and related peptides by skeletal muscle cells in primary culture. *Eur J Biochem* **219,** 43–47.

Baxter C & Roberts E (1961). Elevation of y-aminobutyric acid in brain: Selective inhibition of y-aminobutyric-a-ketoglutaric acid transaminase. *J biol Chem* **236,** 3287.

Bellinger PM (2014). β-Alanine Supplementation for Athletic Performance. *J Strength Cond Res* **28,** 1751–1770.

Bex T, Chung W, Baguet A, Achten E & Derave W (2015). Exercise training and Beta-alanine-induced muscle carnosine loading. *Front Nutr* **2,** 13.

Bex T, Chung W, Baguet A, Stegen S, Stautemas J, Achten E & Derave W (2014). Muscle carnosine loading by beta-alanine supplementation is more pronounced in trained vs. untrained muscles. *J Appl Physiol* **116,** 204–209.

Blancquaert L, Baba S, Kwiatkowski S, Stautemas J, Stegen S, Barbaresi S, Chung W, Boakye A, Hoetker J, Bhatnagar A, Delanghe J, Vanheel B, Veiga-da-Cunha M, Derave W & Everaert I (2016). Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta-alanine transamination. *J Physiol* **594,** 4849–4863.

Blancquaert L, Everaert I & Derave W (2015). Beta-alanine supplementation, muscle carnosine and exercise performance. *Curr Opin Clin Nutr Metab Care* **18,** 63–70.

Boldyrev AA (2012). Carnosine : New Concept for the Function of an Old Molecule. *Biochem* **77,** 313–326.

Boldyrev AA, Aldini G & Derave W (2013). Physiology and pathophysiology of carnosine.

Physiol Rev **93,** 1803–1845.

Boldyrev AA & Severin SE (1990). The histidine-containing dipeptides, carnosine and anserine: Distribution, properties and biological significance. *Adv Enzyme Regul* **30,** 175–188.

Boll M, Foltz M, Rubio-Aliaga I, Kottra G & Daniel H (2002). Functional characterization of two novel mammalian electrogenic proton-dependent amino acid cotransporters. *J Biol Chem* **277,** 22966–22973.

Burke DG, Chilibeck PD, Parise G, Candow DG, Mahoney D & Tarnopolsky M (2003). Effect of Creatine and Weight Training on Muscle Creatine and Performance in Vegetarians. *Med Sci Sports Exerc* **35,** 1946–1955.

Cannon WB (1929). Organization for physiological homeostasis. *Physiol Rev* **IX,** 399–431.

Cederblad G, Carlin JI, Constantin-Teodosiu D, Harper P & Hultman E (1990). Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle. *Anal Biochem* **185,** 274–278.

Chung W, Baguet A, Bex T, Bishop D & Derave W (2014). Doubling of muscle carnosine concentration does not improve laboratory 1-h cycling time trial performance. *Int J Sport Nutr Exerc Metab* **24,** 315–324.

Chung W, Shaw G, Anderson ME, Pyne DB, Saunders PU, Bishop DJ & Burke LM (2012). Effect of 10 week beta-alanine supplementation on competition and training performance in elite swimmers. *Nutrients* **4,** 1441–1453.

Cochran AJ, Percival ME, Thompson S, Gillen JB, MacInnis MJ, Potter M a., Tarnopolsky M a. & Gibala MJ (2015). Beta-Alanine Supplementation Does Not Augment the Skeletal Muscle Adaptive Response to Six Weeks of Sprint Interval Training. *Int J Sport Nutr Exerc Metab*541–549.

Cross a. J, Major JM & Sinha R (2011). Urinary Biomarkers of Meat Consumption. *Cancer Epidemiol Biomarkers Prev* **20,** 1107–1111.

Danaher J, Gerber T, Wellard RM & Stathis CG (2014). The effect of β-alanine and NaHCO3 co-ingestion on buffering capacity and exercise performance with high-intensity exercise in healthy males. *Eur J Appl Physiol* **114,** 1715–1724.

Daniel H (2004). Molecular and integrative physiology of intestinal peptide transport. *Annu Rev Physiol* **66,** 361–384.

Décombaz J, Beaumont M, Vuichoud J, Bouisset F & Stellingwerff T (2012). Effect of slow-release β-alanine tablets on absorption kinetics and paresthesia. *Amino Acids* **43,** 67–76.

DeCourten B, Jakubova M, De Courten MPJ, Kukurova IJ, Vallova S, Krumpolec P, Valkovic L, Kurdiova T, Garzon D, Barbaresi S, Teede HJ, Derave W, Krssak M, Aldini G, Ukropec J & Ukropcova B (2016). Effects of carnosine supplementation on glucose metabolism: Pilot clinical trial. *Obesity* **24,** 1027–1034.

Delanghe J, De Slypere J-P, De Buyzere M, Robbrecht J, Wieme R & Vermeulen A (1989). Normal Reference Values for Creatine, Creatinine, and Carnitine Are Lower in Vegetarians. *Clin Chem* **35,** 1988–1989.

DelFavero S, Roschel H, Solis MY, Hayashi AP, Artioli GG, Otaduy MC, Benatti FB, Harris RC, Wise J a, Leite CC, Pereira RM, de Sá-Pinto AL, Lancha-Junior AH & Gualano B (2012). Beta-alanine (Carnosyn^{TM}) supplementation in elderly subjects (60-80 years): effects on muscle carnosine content and physical capacity. *Amino Acids* **43,** 49–56.

Derave W, Everaert I, Beeckman S & Baguet A (2010). Muscle carnosine metabolism and beta-alanine supplementation in relation to exercise and training. *Sports Med* **40,** 247–263.

Derave W, Ozdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, Wise J a & Achten E (2007). Beta-alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *J Appl Physiol* **103,** 1736–1743.

Dragsted LO (2010). Biomarkers of meat intake and the application of nutrigenomics. *Meat Sci* **84,** 301–307.

Drozak J, Piecuch M, Poleszak O, Kozlowski P, Chrobok L, Baelde HJ & De Heer E (2015). UPF0586 protein C9orf41 homolog is anserine-producing methyltransferase. *J Biol Chem* **290,** 17190–17205.

Drozak J, Veiga-da-Cunha M, Vertommen D, Stroobant V & Van Schaftingen E (2010). Molecular identification of carnosine synthase as ATP-grasp domain-containing protein 1 (ATPGD1). *J Biol Chem* **285,** 9346–9356.

Drummond MJ, Fry CS, Glynn EL, Timmerman KL, Dickinson JM, Walker DK, Gundermann DM, Volpi E & Rasmussen BB (2011). Skeletal muscle amino acid transporter expression is increased in young and older adults following resistance exercise. *J Appl Physiol* **111,** 135–142.

Drummond MJ, Glynn EL, Fry CS, Timmerman KL, Volpi E, Rasmussen BB, Mj D, El G, Cs F, Kl T & Volpi E (2010). An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* **298,** 1011–1018.

Dunnett M & Harris RC (1999). Influence of oral -alanine and L-histidine supplementation

on the carnosine content of the gluteus medies. *Equine Vet J* **30,** 499–504.

Dursun A (2000). Carnitinuria in rickets due to vitamin D deficiency. *Turk J Pediatr* **42,** 278–280.

Dutka TL & Lamb GD (2004). Effect of carnosine on excitation-contraction coupling in mechanically-skinned rat skeletal muscle. *J Muscle Res Cell Motil* **25,** 203–213.

Dutka TL, Lamboley CR, McKenna MJ, Murphy RM & Lamb GD (2012). Effects of carnosine on contractile apparatus Ca^{2+} sensitivity and sarcoplasmic reticulum Ca^{2+} release in human skeletal muscle fibers. *J Appl Physiol* **112,** 728–736.

Endo Y, Tabata T, Kuroda H, Tadano T, Matsushima K & Watanabe M (1998). Induction of histidine decarboxylase in skeletal muscle in mice by electrical stimulation, prolonged walking and interleukin-1. *J Physiol* **509,** 587–598.

Everaert I, Mooyaart A, Baguet A, Zutinic A, Baelde H, Achten E, Taes Y, De Heer E & Derave W (2011). Vegetarianism, female gender and increasing age, but not CNDP1 genotype, are associated with reduced muscle carnosine levels in humans. *Amino Acids* **40,** 1221–1229.

Everaert I, De Naeyer H, Taes Y & Derave W (2013*a*). Gene expression of carnosine-related enzymes and transporters in skeletal muscle. *Eur J Appl Physiol* **113,** 1169–1179.

Everaert I, Stegen S, Vanheel B, Taes Y & Derave W (2013*b*). Effect of beta-alanine and carnosine supplementation on muscle contractility in mice. *Med Sci Sports Exerc* **45,** 43–51.

Feng RN, Niu YC, Sun XW, Li Q, Zhao C, Wang C, Guo FC, Sun CH & Li Y (2013). Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: A randomised controlled trial. *Diabetologia* **56,** 985–994.

Fritz IB & Marquis NR (1965). The role of acylcarnitine esters and carnitine palmityltransferase in the transport of fatty acyl groups across mitochondrial membranes. *Proc Natl Acad Sci U S A* **54,** 1226–1233.

Gale K & Iadarola M (1980). Seizure protection and increased nerve-terminal GABA: delayed effects of GABA transaminase inhibition. *Science (80-)* **208,** 288–291.

Gardner BYMLG, Illingworth KM, Kelleher J & Wood D (1991). Intestinal Absorption of the Intact Peptide Carnosine in Man, and Comparison with Intestinal Permeability to Lactulose. *J Physiol* **439,** 411–422.

van Gelder N (1966). The effect of aminooxyacetic acid on the metabolism of γ-

aminobutyric acid in brain. *Biochem Pharmacol* **15,** 533–539.

Greene S, Margolis F, Grillo M & Fisher H (1984). Enhanced carnosine (B-alanyl-L-Histidine) breakdown and histamine metabolism following treatment with compound 48/80. *Eur J Pharmacol* **99,** 79–84.

Gross M, Boesch C, Bolliger CS, Norman B, Gustafsson T, Hoppeler H & Vogt M (2014). Effects of beta-alanine supplementation and interval training on physiological determinants of severe exercise performance. *Eur J Appl Physiol* **114,** 221–234.

Gualano B, Rawson ES, Candow DG & Chilibeck PD (2016). Creatine supplementation in the aging population: effects on skeletal muscle, bone and brain. *Amino Acids*1–13.

Han X, Patters AB, Jones DP, Zelikovic I & Chesney RW (2006). The taurine transporter: Mechanisms of regulation. *Acta Physiol* **187,** 61–73.

Harris R, Hultman E & Nordesjö L (1974). Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest* **33,** 109–120.

Harris RC, Dunnett M & Greenhaff PL (1998). Carnosine and taurine contents in individual fibres of human vastus lateralis muscle. *J Sports Sci* **16,** 639–643.

Harris RC, Jones G, Hill C, Kendrick IP, Boobis L, Kim C, Kim H, Dang VH, Edge J & Wise J (2007). The Carnosine Content of Vastus Lateralis in Vegetarians and Omnivores. *FASEB J* **21,** A944.

Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill C a, Sale C & Wise J a (2006). The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. *Amino Acids* **30,** 279–289.

Hatazawa Y, Senoo N, Tadaishi M, Ogawa Y, Ezaki O, Kamei Y & Miura S (2015). Metabolomic Analysis of the Skeletal Muscle of Mice Overexpressing PGC-1α. *PLoS One* **10,** e0129084.

Haug A, Rodbotten R, Mydland LT & Christophersen OA (2008). Increased broiler muscle carnosine and anserine following histidine supplementation of commercial broiler feed concentrate. *Acta Agric Scand Sect A-Animal Sci* **58,** 71–77.

Hayaishi O, Nishizuka Y, Tatibana M, Takeshita M & Kuno S (1961). Enzymatic studies on the metabolism of β-alanine. *J Biol Chem* **236,** 781–790.

Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK & Wise JA (2007). Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. *Amino Acids* **32,** 225–233.

Hipkiss AR (2005). Glycation, ageing and carnosine: are carnivorous diets beneficial? *Mech Ageing Dev* **126,** 1034–1039.

Hobson RM, Saunders B, Ball G, Harris RC & Sale C (2012). Effects of β-alanine supplementation on exercise performance: a meta-analysis. *Amino Acids* **43,** 25–37.

Hoffman JR, Ratamess NA, Faigenbaum AD, Ross R, Kang J, Stout JR & Wise JA (2008). Short-duration β-alanine supplementation increases training volume and reduces subjective feelings of fatigue in college football players. *Nutr Res* **28,** 31–35.

Horinishi H, Grillo M & Margolis F (1978). Purification and characterization of carnosine synthetase from mouse olfactory bulbs. *J Neurochem* **31,** 909–919.

Horváth V & Wanders R (1995). Aminooxy acetic acid: a selective inhibitor of alanine: glyoxylate aminotransferase and its use in the diagnosis of primary hyperoxaluria type I. *Clin Chim acta* **243,** 105–114.

Howard J, Cooper B, White H, Soroko F & Maxwell R (1980). A role for GABA in the control of ingestive behavior in rats: effects of ethanolamine-O-sulfate and muscimol. *Brain Res Bull* **5,** 595–599.

Hultman E, Söderlund K, Timmons JA, Cederblad G & Greenhaff PL (1996). Muscle creatine loading in men. *J Appl Physiol* **81,** 232–237.

Ito S, Ohyama T, Kontani Y, Matsuda K, Fujimoto Sakata S & Tamaki N (2001). Influence of dietary protein levels on beta-alanine aminotransferase expression and activity in rats. *J Nutr Sci Vitaminol* **47,** 275–282.

Jaeken J, Casaer P & Cock P De (1984). Gamma-aminobutyric acid-transaminase deficiency: a newly recognized inborn error of neurotransmitter metabolism. *Neuropediatrics* **15,** 165–169.

Jeon SG, Bahn JH, Jang JS, Park J, Kwon OS, Cho SW & Choi SY (2000). Human brain GABA transaminase tissue distribution and molecular expression. *Eur J Biochem* **267,** 5601–5607.

John R, Charteris A & Fowler L (1978). The reaction of amino-oxyacetate with pyridoxal phosphate-dependent enzymes. *Biochem J* **171,** 771–779.

Joint WHO/FAO/UNU Expert Consultation. (2007). Protein and amino acid requirements in human nutrition. *WHO Tech Rep Ser*1–265.

Jones AW (2016). Perspectives in Drug Development and Clinical Pharmacology: The Discovery of Histamine H 1 and H 2 Antagonists. *Clin Pharmacol Drug Dev* **5,** 5–12.

Jung M, Lippert B, Metcalf B, Bohlen P & Schechter P (1977). γ-vinyl GABA (4-amino-hex-

5-enoic acid), a new selective irreversible inhibitor of GABA-T: effects on brain GABA metabolism in mice. *J Neurochem* **29,** 797–802.

Kai S, Watanabe G, Kubota M, Kadowaki M & Fujimura S (2015). Effect of dietary histidine on contents of carnosine and anserine in muscles of broilers. *Anim Sci J* **86,** 541–546.

Kalyankar G & Meister A (1959). Enzymatic synthesis of carnosine and related β-alanyl and ?aminobutyryl peptides. *JBiolChem* **234,** 3210–3218.

Kamal M a, Jiang H, Hu Y, Keep RF & Smith DE (2009). Influence of genetic knockout of Pept2 on the in vivo disposition of endogenous and exogenous carnosine in wild-type and Pept2 null mice. *Am J Physiol Regul Integr Comp Physiol* **296,** R986–R991.

Kaur H, Kumar C, Junot C, Tolendano MB & Bachhawat AK (2009). Dug1p is a Cys-Gly peptidase of the γ-glutamyl cycle of Saccharomyces cerevisiae and represents a novel family of Cys-Gly peptidases. *J Biol Chem* **284,** 14493–14502.

Kendrick IP, Harris RC, Kim HJ, Kim CK, Dang VH, Lam TQ, Bui TT, Smith M & Wise J a (2008). The effects of 10 weeks of resistance training combined with beta-alanine supplementation on whole body strength, force production, muscular endurance and body composition. *Amino Acids* **34,** 547–554.

Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT & Wise JA (2009). The effect of 4 weeks beta-alanine supplementation and isokinetic training on carnosine concentrations in type I and II human skeletal muscle fibres. *Eur J Appl Physiol* **106,** 131–138.

Kimball SR & Jefferson LS (2002). Control of protein synthesis by amino acid availability. *Curr Opin Clin Nutr Metab Care* **5,** 63–67.

Kish S, Perry T & Hansen S (1979). Regional distribition of homocarnosine, homocarnosine-carnosine synthetase and homocarnosinase in human brain. *J Neurochem* **32,** 1629–1636.

Kohen R & Yamamoto Y (1988). Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proc …* **85,** 3175–3179.

Kontani Y, Kawasaki S, Kaneko M, Matsuda K, Fujimoto Sakata S & Tamaki N (1998). Inhibitory effect of ethanol administration on beta-alanine-2-oxoglutarate aminotransferase (GABA aminotransferase) in disulfiram-pretreated rats. *J Nutr Sci Vitaminol* **44,** 165–176.

Krajčovičová-Kudláčková M, Šimončič R, Béderová A, Babinská K & Béder I (2000). Correlation of carnitine levels to methionine and lysine intake. *Physiol Res* **49,** 399–402.

Kralik G, Sak-Bosnar M, Kralik Z, Galovic O, Grcevic M & Kralik I (2015). Effect of β-alanine and L-histidine on concentration of carnosine in muscle tissue and oxidative stability of chicken meat. *Poljoprivreda/Agriculture* **21,** 190–194.

van Kuilenburg ABP, Meinsma R, Beke E, Assmann B, Ribes A, Lorente I, Busch R, Mayatepek E, Abeling NGGM, van Cruchten A, Stroomer AEM, van Lenthe H, Zoetekouw L, Kulik W, Hoffmann GF, Voit T, Wevers RA, Rutsch F & van Gennip AH (2004). β-Ureidopropionase deficiency: An inborn error of pyrimidine degradation associated with neurological abnormalities. *Hum Mol Genet* **13,** 2793–2801.

Kurozumi Y, Abe T, Yao W & Ubuka T (1999). Experimental beta-alaninuria induced by (aminooxy) acetate. *Acta Med Okayama* **53,** 13–18.

Lee M, Nishikimi M, Inoue M, Muragaki Y & Ooshima A (1999). Specific Expression of Alanine-Glyoxylate Aminotransferase 2 in the Epithelial Cells of Henle's Loop. *Nephron* **83,** 184–185.

Leitzmann C (2005). Vegetarian diets: what are the advantages? *Forum Nutr* **57,** 147–156.

Lenney JF, Peppers SC, Kucera-orallo CM & George RP (1985). Characterization of human tissue carnosinase. *Biochem J* **228,** 653–660.

Lippert B, Metcalf BW, Michel JJUNG & Casara P (1977). 4-Amino-hex-5-enoic Acid , a Selective Catalytic Inhibitor of 4-Aminobutyric-Acid Aminotransferase in Mammalian Brain. *Eur J Biochem* **74,** 441–445.

Liu P, Ge X, Ding H, Jiang H, Christensen BM & Li J (2012*a*). Role of glutamate decarboxylase-like protein 1 (GADL1) in taurine biosynthesis. *J Biol Chem* **287,** 40898–40906.

Liu Q, Sikand P, Ma C, Tang Z, Han L, Li Z, Sun S, LaMotte R & X D (2012*b*). Mechanisms ot itch evoked by β-alanine. *J Neurosci* **32,** 14532–14537.

Lombard KA, Olson L, Nelson SE & Rebouche CJ (1989). Carnitine status of lactoovovegetarians and strict vegetarian adults and children. *Am J Clin Nutr* **50,** 301–306.

Löscher W & Frey H (1978). Aminooxyacetic acid: correlation between biochemical effects, anticonvulsant action and toxicity in mice. *Biochem Pharmacol* **27,** 103–108.

Lukaszuk JM, Robertson RJ, Arch JE, Moore GE, Yaw KM, Kelley DE, Rubin JT & Moyna NM (2002). Effect of creatine supplementation and a lacto-ovo-vegetarian diet on muscle creatine concentration. *Int J Sport Nutr Exerc Metab* **12,** 336–348.

Mannion a F, Jakeman PM & Willan PL (1995). Skeletal muscle buffer value, fibre type distribution and high intensity exercise performance in man. *Exp Physiol* **80,** 89–101.

Matthews MM, Trauts TW & Carolina N (1987). Regulation of N-carbomoyl-β-alanine amidohydrolase, the terminal enzyme in pyrimidine catabolsim, by ligand-induced change in polymerization. *J Biol Chem* **262,** 7232–7237.

McCarty MF (2004). Sub-optimal taurine status may promote platelet hyperaggregability in vegetarians. *Med Hypotheses* **63,** 426–433.

McEvoy CT, Temple N & Woodside J V (2012). Vegetarian diets, low-meat diets and health: a review. *Public Health Nutr* **15,** 2287–2294.

Metzner L, Neubert K & Brandsch M (2006). Substrate specificity of the amino acid transporter PAT1. *Amino Acids* **31,** 111–117.

Miyaji T, Sato M, Maemura H, Takahata Y & Morimatsu F (2012). Expression profiles of carnosine synthesis-related genes in mice after ingestion of carnosine or ß-alanine. *J Int Soc Sports Nutr* **9,** 15.

Mong MC, Chao CY & Yin MC (2011). Histidine and carnosine alleviated hepatic steatosis in mice consumed high saturated fat diet. *Eur J Pharmacol* **653,** 82–88.

Ng R & Marshall F (1978). Regional and subcellular distribution of homocarnosine-carnosine synthetase in the central nervous system of rats. *J Neurochem* **30,** 187–190.

Niijima-Yaoita F, Tsuchiya M, Ohtsu H, Yanai K, Sugawara S, Endo Y & Tadano T (2012). Roles of histamine in exercise-induced fatigue: favouring endurance and protecting against exhaustion. *Biol Pharm Bull* **35,** 91–97.

Novakova K, Kummer O, Bouitbir J, Stoffel SD, Hoerler-Koerner U, Bodmer M, Roberts P, Urwyler A, Ehrsam R & Krähenbühl S (2016). Effect of l-carnitine supplementation on the body carnitine pool, skeletal muscle energy metabolism and physical performance in male vegetarians. *Eur J Nutr* **55,** 207–217.

O'Dowd JJ, Robins DJ & Miller DJ (1988). Detection, characterisation, and quantification of carnosine and other histidyl derivatives in cardiac and skeletal muscle. *Biochim Biophys Acta* **967,** 241–249.

Okubo H & Sasaki S (2005). Histidine intake may negatively correlate with energy intake in human: a cross-sectional study in Japanese female students aged 18 years. *J Nutr Sci Vitaminol (Tokyo)* **51,** 329–334.

Orlich MJ, Singh PN, Sabaté J, Jaceldo-Siegl K, Fan J, Knutsen S, Beeson WL & Fraser GE (2013). Vegetarian dietary patterns and mortality in Adventist Health Study 2. *JAMA Intern Med* **173,** 1230–1238.

Ozdemir MS, Reyngoudt H, De Deene Y, Sazak HS, Fieremans E, Delputte S, D'Asseler Y,

Derave W, Lemahieu I & Achten E (2007). Absolute quantification of carnosine in human calf muscle by proton magnetic resonance spectroscopy. *Phys Med Biol* **52,** 6781–6794.

Pandya V, Ekka MK, Dutta RK & Kumaran S (2011). Mass spectrometry assay for studying kinetic properties of dipeptidases: Characterization of human and yeast dipeptidases. *Anal Biochem* **418,** 134–142.

Park S, Kim C & Namgung N (2013). Effects of dietary supplementation of histidine, β-alanine, magnesium oxide, and blood meal on carnosine and anserine concentrations of broiler breast meat. *J Poult Sci* **50,** 251–256.

Park S, Kim DS & Kang S (2016). Vitamin D deficiency impairs glucose-stimulated insulin secretion and increases insulin resistance by reducing PPAR-ɣ expression in nonobese Type 2 diabetic rats. *J Nutr Biochem* **27,** 257–265.

Peñafiel R, Ruzafa C, Monserrat F & Cremades a (2004). Gender-related differences in carnosine, anserine and lysine content of murine skeletal muscle. *Amino Acids* **26,** 53–58.

Perry T, Wright J, Hansen S, Allan B, Baird P & MacLeod P (1980). Failure of aminooxyacetic acid therapy in Huntington disease. *Neurology* **30,** 31–44.

Peters V, Kebbewar M, Jansen EW, Jakobs C, Riedl E, Koeppel H, Frey D, Adelmann K, Klingbeil K, MacK M, Hoffmann GF, Janssen B, Zschocke J & Yard BA (2010). Relevance of allosteric conformations and homocarnosine concentration on carnosinase activity. *Amino Acids* **38,** 1607–1615.

Peters V, Klessens CQF, Baelde HJ, Singler B, Veraar KAM, Zutinic A, Drozak J, Zschocke J, Schmitt CP & De Heer E (2015). Intrinsic carnosine metabolism in the human kidney. *Amino Acids* **47,** 2541–2550.

Peters V, Schmitt CP, Zschocke J, Gross M-L, Brismar K & Forsberg E (2012). Carnosine treatment largely prevents alterations of renal carnosine metabolism in diabetic mice. *Amino Acids* **42,** 2411–2416.

Petroff O a, Hyder F, Collins T, Mattson RH & Rothman DL (1999). Acute effects of vigabatrin on brain GABA and homocarnosine in patients with complex partial seizures. *Epilepsia* **40,** 958–964.

Petroff O a, Mattson RH, Behar KL, Hyder F & Rothman DL (1998). Vigabatrin increases human brain homocarnosine and improves seizure control. *Ann Neurol* **44,** 948–952.

Pfister F, Riedl E & Wang Q (2011). Oral carnosine supplementation prevents vascular damage in experimental diabetic retinopathy. *Cell Physiol …*. Available at: http://www.karger.com/Article/Abstract/331721 [Accessed August 1, 2013].

Phillips SM (2004). Protein requirements and supplementation in strength sports. *Nutrition* **20,** 689–695.

Pierno S, Liantonio A, Camerino GM, De Bellis M, Cannone M, Gramegna G, Scaramuzzi A, Simonetti S, Nicchia GP, Basco D, Svelto M, Desaphy JF & Camerino DC (2012). Potential benefits of taurine in the prevention of skeletal muscle impairment induced by disuse in the hindlimb-unloaded rat. *Amino Acids* **43,** 431–445.

Pihl A & Fritzson P (1955). The catabolism of C14-labeled β -alanine in the intact rat. *J Biol Chem* **215,** 345–351.

Pinto CL, Botelho PB, Pimentel GD, Campos-Ferraz PL & Mota JF (2016). Creatine supplementation and glycemic control: a systematic review. *Amino Acids*; DOI: 10.1007/s00726-016-2277-1.

Quinn M & Fisher H (1977). Effect of dietary histidine deprivation in two rat strains on hemoglobin and tissue concentrations of histidine-containing dipeptides. *J Nutr* **107,** 2044–2054.

Rebouche J, Lombard A & Chenard A (1993). Renal adaptation to dietary carnitine in humans. *Am J Clin Nutr* **58,** 660–665.

Reeves R & Barbour G (1977). Failure of histidine supplementation to improve anemia in chronic dialysis patients. *Am J Clin Nutr* **30,** 579–581.

Riedl E, Pfister F, Braunagel M, Brinkk??tter P, Sternik P, Deinzer M, Bakker SJL, Henning RH, Van Den Born J, Kr??mer BK, Navis G, Hammes HP, Yard B & Koeppel H (2011). Carnosine prevents apoptosis of glomerular cells and podocyte loss in stz diabetic rats. *Cell Physiol Biochem* **28,** 279–288.

Rodionov RN, Jarzebska N, Weiss N & Lentz SR (2014). AGXT2: a promiscuous aminotransferase. *Trends Pharmacol Sci* **35,** 575–582.

Romero SA, Hocker AD, Mangum JE, Luttrell MJ, Turnbull DW, Struck AJ, Ely MR, Sieck DC, Dreyer HC & Halliwill JR (2016). Evidence of a broad histamine footprint on the human exercise transcriptome. *J Physiol*In press.

Sadikali F, Darwish R & Watson WC (1975). Carnosinase activity of human gastrointestinal mucosa. *Gut* **16,** 585–589.

Saunders B, Elliot-Sale K, Artioli GG, Swinton PA, Dolan E, Roschel H, Sale C & Gualano B (2016). β-alanine supplementation to improve exercise capacity and performance: a systematic review and meta-analysis. *Br J Sports Med*; DOI: 10.1136.

Scriver C, Pueschel S & Davies E (1966). Hyper-β-alaninemia associated with β-aminoaciduria and γ-aminobutyricaciduria, somnolence and seizures. *N Engl J Med*

274, 635–643.

Seiler S, Koves T, Gooding J, Wong K, Stevens R, Ilkayeva O, Wittmann A, DeBalsi K, Davies M, Lindeboom L, Schrauwen P, Schrauwen-Hinderling V & Muoio D (2015). Carnitine Acetyltransferase Mitigates Metabolic Inertia and Muscle Fatigue During Exercise. *Cell Metab* **22,** 65–76.

Spriet LL & Whitfield J (2015). Taurine and skeletal muscle function. *Curr Opin Clin Nutr Metab Care* **18,** 96–101.

Stegen S, Bex T, Vervaet C, Vanhee L, Achten E & Derave W (2014). The beta-alanine dose for maintaining moderately elevated muscle carnosine levels. *Med Sci Sports Exerc* **46,** 1426–1432.

Stegen S, Blancquaert L, Everaert I, Bex T, Taes Y, Calders P, Achten E & Derave W (2013*a*). Meal and beta-alanine coingestion enhances muscle carnosine loading. *Med Sci Sports Exerc* **45,** 1478–1485.

Stegen S, Blancquaert L, Everaert I, Bex T, Taes Y, Calders P, Achten E & Derave W (2013*b*). Meal and Beta-Alanine Coingestion Enhances Muscle Carnosine Loading. *Med Sci Sports Exerc*; DOI: 10.1249/MSS.0b013e31828ab073.

Stegen S, Stegen B, Aldini G, Altomare A, Cannizzaro L, Orioli M, Gerlo S, Deldicque L, Ramaekers M, Hespel P & Derave W (2015). Plasma carnosine, but not muscle carnosine, attenuates high-fat diet-induced metabolic stress. *Appl Physiol Nutr Metab* **40,** 868–876.

Steiber A, Kerner J & Hoppel CL (2004). Carnitine: A nutritional, biosynthetic, and functional perspective. *Mol Aspects Med* **25,** 455–473.

Stellingwerff T, Anwander H, Egger A, Buehler T, Kreis R, Decombaz J & Boesch C (2012*a*). Effect of two β-alanine dosing protocols on muscle carnosine synthesis and washout. *Amino Acids* **42,** 2461–2472.

Stellingwerff T, Decombaz J, Harris RC & Boesch C (2012*b*). Optimizing human in vivo dosing and delivery of β-alanine supplements for muscle carnosine synthesis. *Amino Acids* **43,** 57–65.

Stephens FB, Marimuthu K, Cheng Y, Patel N, Constantin D, Simpson EJ & Greenhaff PL (2011). Vegetarians have a reduced skeletal muscle carnitine transport capacity. *Am J Clin Nutr* **94,** 938–944.

Stifel F & Herman R (1972). Is histidine an essential amino acid in man? *Am J Clin Nutr* **25,** 182–185.

Stout JR, Cramer JT, Zoeller RF, Torok D, Costa P, Hoffman JR, Harris RC & O'Kroy J (2007).

Effects of beta-alanine supplementation on the onset of neuromuscular fatigue and ventilatory threshold in women. *Amino Acids* **32,** 381–386.

Suhre K et al. (2011). A genome-wide association study of metabolic traits in human urine. *Nat Genet* **43,** 565–569.

Swietach P, Leem C-H, Spitzer KW & Vaughan-Jones RD (2014). Pumping Ca2+ ions up H+ gradients: a cytoplasmic Ca2+/H+ exchanger without a membrane. *J Physiol* **00,** 1–10.

Swietach P, Youm J-B, Saegusa N, Leem C-H, Spitzer KW & Vaughan-Jones RD (2013). Coupled Ca2+/H+ transport by cytoplasmic buffers regulates local Ca2+ and H+ ion signaling. *Proc Natl Acad Sci U S A* **110,** E2064–E2073.

Tamaki N, Funatsuka A, Fujimoto S & Hama T (1984). The utilization of carnosine in rats fed on a histidine-free diet and its effect on the levels of tissue histidine and carnosine. *J Nutr Sci Vitaminol (Tokyo)* **30,** 541–551.

Tamaki N, Funatsuka A, Wakabayashi M & Hama T (1977). Effect of histidine-free and-excess diets on anserine and carnosine contents in rat gastrocnemius muscle. *J Nutr Sci Vitaminol (Tokyo)* **23,** 331–340.

Tamaki N, Kaneko M, Mizota C, Kikugawa M & Fujimoto S (1990). Purification, characterization and inhibition of d-3-aminoisobutyrate aminotransferase from the rat liver. *Eur J Biochem* **189,** 39–45.

Tamaki N, Morioka S, Ikeda T, Harada M & Hama T (1980). Biosynthesis and degradation of carnosine and turnover rate of its constituent amino acids in rats. *J Nutr Sci Vitaminol* **26,** 127–139.

Tantamango-Bartley Y, Jaceldo-Siegl K, Fan J & Fraser G (2013). Vegetarian diets and the incidence of cancer in a low-risk population. *Cancer Epidemiol Biomarkers Prev* **22,** 286–294.

Teufel M, Saudek V, Ledig J-P, Bernhardt A, Boularand S, Carreau A, Cairns NJ, Carter C, Cowley DJ, Duverger D, Ganzhorn AJ, Guenet C, Heintzelmann B, Laucher V, Sauvage C & Smirnova T (2003). Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* **278,** 6521–6531.

Traut TW (2000). *β-Alanine Synthase an Enzyme Involved in Catabolism of Uracil and Thymine*. Elsevier Masson SAS. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0076687900242493.

Twycross-Lewis R, Kilduff LP, Wang G & Pitsiladis YP (2016). The effects of creatine supplementation on thermoregulation and physical (cognitive) performance: a review and future prospects. *Amino Acids*1–13.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3,** 0034.1–0034.11.

Veiga-da-Cunha M, Chevalier N, Stroobant V, Vertommen D & Van Schaftingen E (2014). Metabolite proofreading in carnosine and homocarnosine synthesis: molecular identification of PM20D2 as β-alanyl-lysine dipeptidase. *J Biol Chem* **289,** 19726–19736.

Wall BT, Stephens FB, Constantin-Teodosiu D, Marimuthu K, Macdonald IA & Greenhaff PL (2011). Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. *J Physiol J Physiol J Physiol* **5894,** 963–973.

Wallach D (1961). Studies on the GABA pathway. *Biochem Pharmacol* **5,** 323–331.

Wilson K, Kirwan J, Derave W, Hess J, Zhang G, Brunengraber H & Tochtrop G (2016). Metabolism of beta-alanine in rat liver: degradation to acetyl-CoA and carboxylation to 2-(aminomethyl)-malonate. *Present poster JESIUM Congr*.

Wyss M & Kaddurah-Daouk R (2000). Creatine and Creatinine Metabolism. *Physiol Rev* **80,** 1107–1213.

Yoo DY, Choi JH, Kim W, Yoo K-Y, Lee CH, Yoon YS, Won M-H & Hwang IK (2011). Effects of Melissa officinalis L. (lemon balm) extract on neurogenesis associated with serum corticosterone and GABA in the mouse dentate gyrus. *Neurochem Res* **36,** 250–257.

Yoshino Y, Abe M, Numata S, Ochi S, Mori Y, Ishimaru T, Kinoshita M, Umehara H, Yamazaki K, Mori T, Ohmori T & Ueno S (2014). Missense variants of the alanine:glyoxylate aminotransferase 2 gene are not associated with Japanese schizophrenia patients. *Prog Neuropsychopharmacol Biol Psychiatry* **53,** 137–141.

V

Publications

A1

Publications included in this thesis:

Blancquaert L, Baba SP, Kwiatkowski S, Stautemas J, Stegen S, Barbaresi S, Chung W, Boakye AA, Hoetker JD, Bhatnagar A, Delanghe K, Vanheel B, Veiga-da-cunha M, Derave W, Everaert I. Carnosine and anserine homeostasis in skeletal muscle and heart is controlled by beta-alanine transamination. *J Physiol* 594(17):4849-63, 2016.

Blancquaert L, Everaert I, Missinne M, Baguet A, Stegen S, Volkaert A, Petrovic M, Vervaet C, Achten E, De Maeyer M, De Henauw S, Derave W. Effects of histidine and beta-alanine supplementation on human muscle carnosine storage. *Med Sci Sports Exerc* (accepted for publication).

Blancquaert L, Baguet A, Bex T, Volkaert A, Everaert I, Delanghe J, Petrovic M, Vervaet C, Achten E, Constantin-Teodosio Dumitru, Greenhaff P, Derave W. Body creatine, but not carnitine and carnosine stores, decline by a 6-month vegetarian diet In omnivorous women. *Am J Clin Nutr*. In preparation.

Other publications:

Blancquaert L, Everaert I, Derave W. Beta-alanine supplementation, muscle carnosine and exercise performance. *Curr Opin Clin Nutr Metab Care* 18(1):63-70, 2015.

Stegen S, **Blancquaert L**, Everaert I, Bex T, Taes Y, Calders P, Achten E, Derave W. Meal and beta-alanine coingestion enhances muscle carnosine loading. *Med Sci Sports Exerc* 45(8):1478-85, 2013.

A3

Blancquaert L, Derave W. Beta-alanine en sportprestaties. Ortho-reumato 13(6):6-10, 2015

C1-C3

21th annual European College of Sport Sciences Congress – Vienna, Austria – 6-9 July, 2016

Blancquaert L, Everaert I, Derave W. Fixing the metabolic leak in the inefficient muscle carnosine loading scheme by oral beta-alanine supplementation.

(oral presentation)

Research Day – Ghent, Belgium – 16 March, 2016

Blancquaert L, Baguet A, Derave W. Effect of a long term vegetarian diet on body creatine, carnitine and carnosine stores in omnivorous women.

(bullet presentation)

Carnosine Symposium – Gabicce Mare, Italy – 7-8 June, 2015

Blancquaert L, Everaert I, Derave W. Inhibiting beta-alanine transaminase: a way to promote muscle histidine-containing dipeptide loading in mice?

(oral presentation)

19th Symposium of the Flemish Society for Kinesiology – Antwerp, Belgium – 12 December, 2014

Blancquaert L, Everaert I, Baguet A, Derave W. Effect of beta-alanine and L-histidine supplementation on muscle carnosine loading. 2nd place Young Investigator's Award.

(oral presentation)

Interuniversity Doctoral School: State of the Art in Exercise Physiology – Louvain-la-Neuve, Belgium – 5 November, 2014

Blancquaert L, Everaert I, Baguet A, Derave W. Effect of beta-alanine and L-histidine supplementation on muscle carnosine loading.

(oral presentation)

3th International Congress on Carnosine and Anserine – Tokyo, Japan – 5-7 August, 2014

Blancquaert L, Everaert I, Baguet A, Derave W. Effect of beta-alanine and L-histidine supplementation on muscle carnosine loading.

(oral presentation and poster)

3th International Congress on Carnosine and Anserine – Tokyo, Japan – 5-7 August, 2014

Blancquaert L, Chung W, Everaert I, Ghillebert J, Derave W. Inhibiting beta-alanine transaminase: a way to promote muscle histidine-containing dipeptide loading in mice?

(poster presentation)

Interuniversity Doctoral School: State of the Art in Exercise Physiology – Leuven, Belgium – 12 May, 2014

Blancquaert L, Everaert I, Stegen S, Derave W. Gene expression of carnosine-related enzymes and transporters in human skeletal muscle: influence of chronic beta-alanine supplementation.

(oral presentation)

18th annual European College of Sport Sciences Congress – Barcelona, Spain – 26-29 June, 2013

Blancquaert L, Stegen S, Everaert I, Bex T, Taes Y, Vanhee L, Vervaet C, Calders P, Achten E, Derave W. Meal and beta-alanine co-ingestion enhances muscle carnosine loading.

(poster presentation)

VI

Appendices

Appendix 1: professional career Laura Blancquaert

Laura started her studies Physical Education and Movement Sciences at Ghent University in 2007 and graduated her master in 2011 with great honor. After her graduation, she worked as assisting academic staff member during an interim period of three months under supervision of Prof. Wim Derave and she finished her teacher education in the same year. The interim period in the lab of Prof. Derave led to an application for a PhD project for the Reasearch Foundation Flanders, which was succesfully awarded in 2012. During her PhD period, Laura participated in several national and international conferences where she presented her scientific work and she did a 6-week short stay in Louisville (USA) where she learned different analytical techniques. Her scientific work during her PhD has contributed to a number of publications, which can be found in the publication list. Laura has also been teaching practical classes in physiology to undergraduates and has supervised master students during the preparation of their master thesis.