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Department of Movement and Sports Sciences

THE CARNOSINE-CARNOSINASE SYSTEM IN RELATION TO DIABETES

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Acknowledgments – Voorwoord

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Part I

General Introduction

1. INTRO

It is suggested that:

- the molecule carnosine is a potential candidate to counteract the development of diabetes or diabetes-related diseases.

This is based on:

- plentiful in vitro studies focusing on its biochemical and physiological protective characteristics

However, up until now:

- the behavior of endogenous carnosine in a diabetic setting is barely known
- little in vivo studies are present about the beneficial effects of carnosine supplementation

Therefore, this work contains animal and human data to provide:

- insight into the carnosine metabolism and how it is possibly altered in a diabetic state in rodents and humans
- in vivo evidence about the protective effects of carnosine supplementation in a very mild obese-diabetic rat model

At least an interesting topic and worthwhile to investigate!

2. CARNOSINE AS THE FOUNDING MEMBER OF HISTIDINE-CONTAINING DIPEPTIDES

The story of the molecule carnosine starts at the beginning of the 20th century, because of a man called Vladimir Gulewitch. This young Russian chemist, excellently graduated from Moscow State University in 1890, discovered carnosine during his search for unidentified nitrogen-containing non-protein compounds in meat extracts. Gulewitch identified two new molecules present in rather high amounts and called them carnosine and carnitine (from the Latin *carnis* – *meat*) (Gulewitch 1900). He concentrated his work on the substance with the highest amount, i.e. **carnosine**, and in 1911, the structure of carnosine was established (it was found to be the dipeptide beta-alanine-L-histidine)(Gulewitch 1911). This was the starting point of the "Russian contribution" in World biochemistry of the biological activity of carnosine.

Figure 1: Vladimir Gulewitch at his laboratory desk (1929)

In the 1920s, German scientists noticed that muscles of most birds do not contain carnosine, instead its methylated analogue **anserine** (β-alanyl-N-π-methylhistidine) was identified, whereby the name was derived from the Latin word for *goose*, i.e. *anser*. (Ackermann 1929; Tolkatschewskaya 1929).

Thereafter, several other **carnosine related compounds** were identified (figure 2). From the perspective of carnosine, the most common variations include 1) methylation on either the π or τ nitrogen in the imidazole ring of histidine to form respectively anserine (β-alanyl-N-π-methylhistidine) or ophidine (β-alanyl-N-τ-methylhistidine), 2) substitution of beta-alanine by γ-aminobutyric acid (GABA) to form homocarnosine (γ-aminobutyryl-histidine), or 3) acetylation of the terminal amino group of beta-alanine to

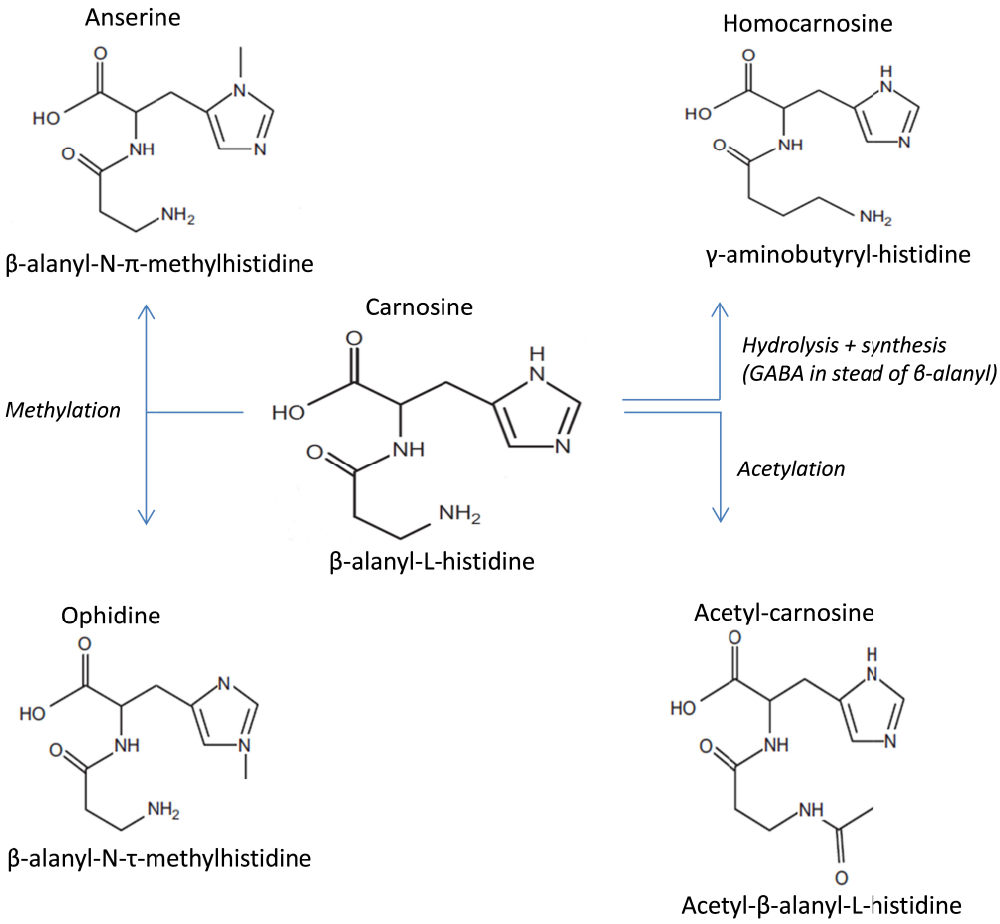

Figure 2. Chemical structure of carnosine and its most common related compounds. The variations are represented by arrows.

form N-acetylcarnosine**. These modifications are species and tissue-specific** (Boldyrev 2013). Species-specific because carnosine is the only compound present in human skeletal muscle, whereas the muscles of almost all other mammals have both carnosine along with its methylated form anserine. Only the muscles of marine diving mammals, such as whales and dolphins, have carnosine along with the methylated form ophidine. Tissue-specific because carnosine and its methylated forms are predominantly located in skeletal muscle, whereas homocarnosine is predominantly present in brain structures. However, the specific function of each of these dipeptides is widely investigated but still not fully understood.

So, carnosine is **the 'founding member'** of a series of compounds, which are also collectively called the **histidine-containing dipeptides** (**HCDs**). These dipeptides consist of a histidine (or a histidine like amino acid) and an atypical amino acid (e.g. beta-alanine or

γ-aminobutyric acid) and they are predominantly present in excitable tissues, such as muscle and nervous tissue (Boldyrev 1990).

In this work, HCDs will refer to carnosine and anserine, as this work will focus predominantly on human and rodent muscles.

3. CARNOSINE – CARNOSINASE METABOLISM IN HUMANS AND RODENTS

Both in humans and rodents, more than 99% of total body carnosine (and also its methylated form anserine for rodents) is present in skeletal muscle. This leads to very high carnosine concentrations of around ~20-30 mmol/kg dry muscle in humans and around ~5-20 mmol/kg dry muscle in rats. Since rats possess anserine as well in concentrations of ~20-40 mmol/kg dry muscle, this leads to a more than double HCD content compared to humans. To date, it is not fully understood whether anserine is synthesized as a result of direct methylation of carnosine by the carnosine-N-methyltransferase (Mcmanus 1962; Bauer 1994) or as a result of the condensation between beta-alanine and π-methyl-L-histidine (Drozak 2010).

For many years, it was suspected that carnosine was only present in skeletal muscle (in millimolar range), in brain (in micromolar range) and in the myocardium of rodents (Aldini 2004). Recent papers demonstrated, however, that it is present in other tissues as well, such as kidney (Kamal 2009; Riedl 2011), liver (Mong 2011), retina (Pfister 2011), spleen (Kamal 2009),…although in very low concentrations. Literature about carnosine content in human tissues other than skeletal muscle is currently lacking, but it is suspected to be absent (Jackson 1996) or limited to the olfactory system in the brain (Petroff 1998).

The two main biochemical pathways are its synthesis from and its breakdown (hydrolysis) into the two constituent amino acids beta-alanine and L-histidine. L-histidine is a proteinogenic and essential α-amino acid. Under normal physiological conditions, the histidine concentration in biological tissues is present in sufficient amounts , except when histidine-free diets are consumed (Tamaki 1977). Beta-alanine is a rather unusual amino acid because it is the only naturally occurring free β-amino acid and it is not used in the biosynthesis of proteins. Beta-alanine is endogenously formed in the liver by degradation

of uracil and thereafter it is secreted into the serum (Traut 2000) or it can be obtained by the ingestion of meat and fish, from the hydrolysis of HCD. Noteworhty, beta-alanine is a component of the essential vitamin B5 (pantothenic acid), which itself is a component of coenzyme A. However, the degradation (or synthesis) of pantothenic acid into (or from) its constituent components (beta-alanine and pantoic acid) does not occur in humans or other animals (Gropper 2009).

In general, carnosine synthesis mainly takes place in skeletal muscles by the enzyme carnosine synthase (Gulewitch 1911; Drozak 2010), whereas its breakdown occurs predominantly in circulation by serum carnosinase (CN1, encoded by the gene CNDP1)(Lenney 1990). However, carnosinase is also present in tissues as non-specific dipeptidase or tissue carnosinase (CN2, encoded by another gene, i.e. CNDP2), which has been demonstrated in kidney, liver, spleen, small intestine and yes, even muscle (Lenney 1985). Noteworthy, the catalytical rate of CN2 in tissues is markedly lower than the catalytical rate of CN1 in serum (Pandya 2011). Indeed, CN2 has a broader substrate specificity then CN1 and the optimum pH for CN2 is 9.5, whereas the pH of tissues do not exceed 7.4 (Lenney 1985; Teufel 2003). The presence of carnosine synthase in other tissues than skeletal muscle in humans is not yet investigated but Miyaji et al. (2012) has detected mRNA levels in brain, liver and kidney in rat tissues, which is not surprising as carnosine was also very recently detected in those tissues, although at very low concentrations (Kamal 2009; Riedl 2011; Pfister 2011).

More in detail, five important pathways in the metabolism of carnosine can be formulated, which are overviewed in figure 3. A first important step **(I)** is the absorption of carnosine from the diet. Carnosine is partly intactly transported across the intestinal cells as carnosine appears in blood when high doses are ingested (Gardner 1991; Everaert 2012). Indeed, PEPT 1 (member of the proton-coupled oligopeptide transporter (POT)-family, which are able to transfer di- and tripeptides across biological membranes) is expressed in the brush border membrane of enterocytes and is able to transfer the HCDs using an inwardly direct proton gradient (Daniel 2004; Geissler 2010). However, it is suggested that low doses of carnosine are mainly hydrolyzed in the enterocytes by CN2

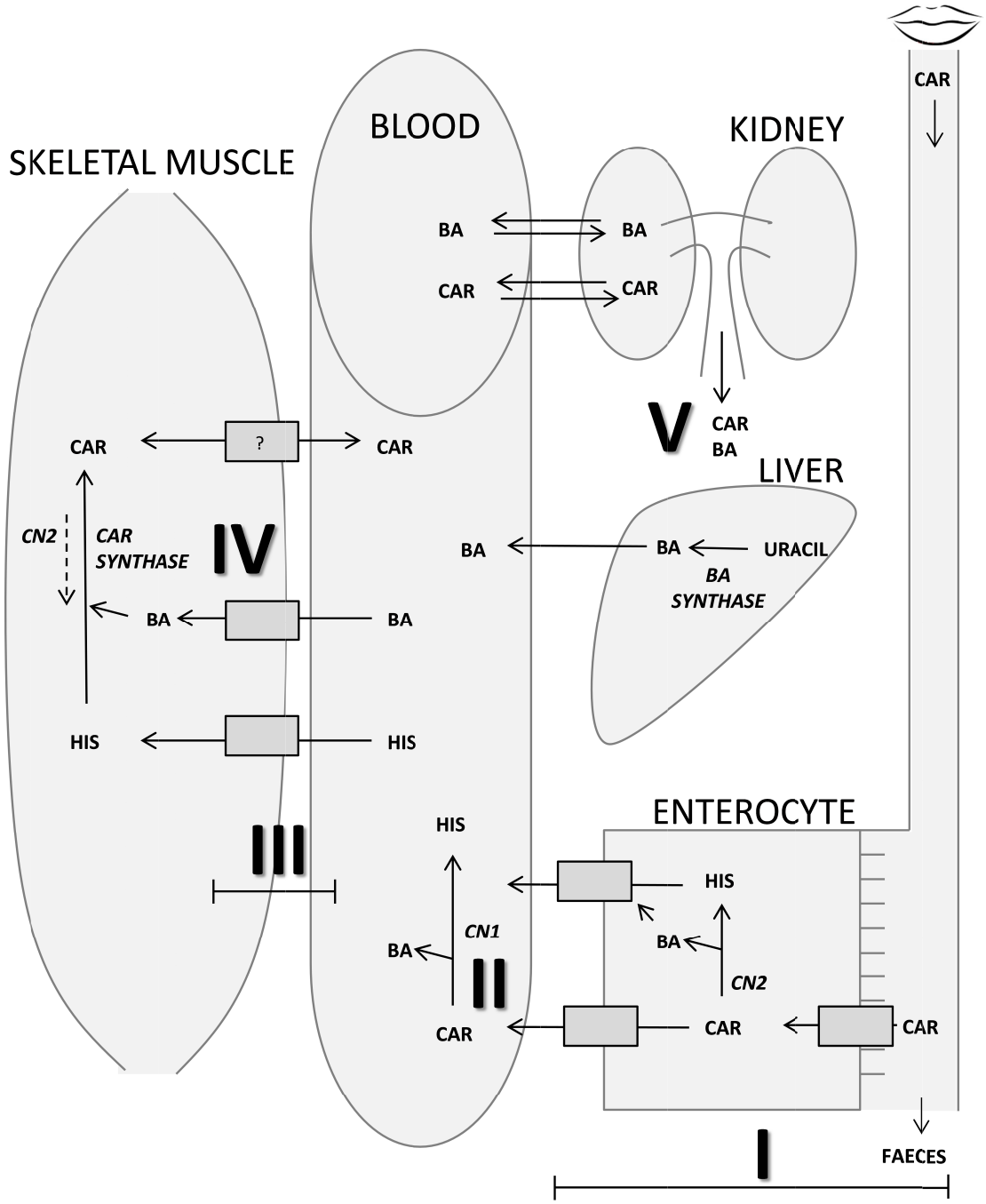

Figure 3. Schematical representation of the five most important pathways in the metabolism of carnosine in humans. I) absorption of carnosine from the diet, II) carnosine is almost immediately hydrolyzed by CN1 in circulation, III) The constituent amino acids (BA and HIS) are transported into the skeletal muscle, IV) Carnosine synthesis, V) carnosine is excreted in urine (only if high doses are consumed). Abbreviations: CAR: carnosine, BA: beta-alanine, HIS: L-histidine, CN1: serum carnosinase, CN2: tissue carnosinase or non-specific dipeptidase.

and that beta-alanine and (methyl)-histidine are subsequently transported into the blood by amino acid transporters (Lenney 1985; Bauchart 2007).

(II) Second, if carnosine reaches the blood (if not hydrolyzed by the enterocytes), it is almost immediately hydrolyzed in the circulation by CN1. Noteworthy, CN1 is very active in humans in the circulation, but it is absent in the circulation of rodents (Jackson 1991; Aldini 2011). In these animals, the catalytical activity of carnosine mainly takes place in the kidneys where CN2 is located (Aldini 2011). So, upon ingestion, plasma carnosine levels are elevated for ~2 hours in rodents, whereas in humans carnosine is often not detectable upon ingestion, or only when high doses are consumed and only in subjects with low activity of serum carnosinase, with a peak around 30 minutes after ingestion (Everaert 2012).

(III) Third, it is clear from above that no or little ingested carnosine is left to be transported into the muscles or other organs. The constituent amino acids are then transported into the skeletal muscle, with histidine being transported by amino acid transporters and beta-alanine mainly by the taurine transporter (TauT) (Harris 2006).

(IV) Since concentrations of beta-alanine in the muscle are very low (< 10 µmol/kg wm)(Harris 2006), compared to histidine (~0.5-1 mmol/kg wm) (Parkhouse 1985), it has several times been concluded that beta-alanine, rather than L-histidine, is the rate-limiting precursor in carnosine synthesis in humans (fourth pathway) (Bakardjiev 1994; Dunnett 1999; Harris 2006). Noteworthy, brains are also characterized with relatively high concentrations of carnosine(-analogues) and the metabolism is quite similar than the skeletal muscle because brain tissues are also equipped with taurine transporters (Fujita 2006) and relative high carnosine synthase activity (Harding 1976). In addition, Murakami et al. (2010) has demonstrated that brain carnosine levels are also enhanced by long-term beta-alanine supplementation in mice (Murakami 2010). However, as said previously, the most common HCD in mammalian brain is the analogue of carnosine ‘homocarnosine’ (Petroff 1998), whereby beta-alanine is replaced by GABA, an important neurotransmitter in the brain (Kish 1979).

(V) Finally, *if* high doses of carnosine are supplemented and *if* carnosine is not (yet) hydrolyzed by CN2 in enterocyts or by CN1 in circulation, *then* carnosine can appear in urine. However, carnosine is mainly reabsorbed from the filtrate and metabolized in the renal cells by CN2.

Tables 1A and 1B summarize the presence or absence of carnosine/anserine and the most important enzymes in the metabolism of carnosine in several tissues in respectively humans and rodents.

Conclusion: HCDs are predominantly and abundantly present in skeletal muscle. The carnosine system in rodents and humans is quite similar, however two important differences can be put forward. First, rodents are characterized by the absence of serum carnosinase. Second, skeletal muscles of rodents also possess the methylated variant anserine. This work will study both humans and rodents (rats/mice).

Table 1A. The presence or absence of carnosine/anserine and the most important enzymes in the metabolism of carnosine in several tissues of **HUMANS.**

	Blood	Skeletal muscle	Brain/CNS	Other organs
Carnosine	**0/+** ✓ Not in fasted state (Gardner 1991; Park 2005; Suzuki 2006; Yeum 2010) ✓ Or nM concentrations (Fonteh 2007; Zhou 2013) ✓ Only after supplementation (Everaert 2012)	**+++** ✓ (Parkhouse 1985; Harris 1990; Mannion 1992)	**+** ✓ Very little (if any) in olfactory bulb (Kish 1979) ✓ Homocarnosine is abundantly present (Kish 1979; Petroff 1998)	**0/?** ✓ (Jackson 1996)
Anserine	**0/+** ✓ Not in fasted state (Suzuki 2006) ✓ Only limited after supplementation (Suzuki 2006; Kubomura 2009)	**0** ✓ (Kohen 1988; Mannion 1992)	**0** ✓ (Jackson 1996)	**0** ✓ (Jackson 1996)
Carnosine synthase	**0**	**+++** ✓ mRNA levels (Baguet 2011b; Everaert 2013)	**+** ✓ Homocarnosine – carnosine synthase activity (Kish 1979)	**?**
CN1	**+++** ✓ (Lenney 1982)	**0** ✓ (Lenney 1982)	**++** ✓ (Teufel 2003)	**0/+** ✓ Absent (Lenney 1982) <-> mRNA in liver, kidney (Teufel 2003; Janssen 2005)
CN2	**0** ✓ (Lenney 1982; Lenney 1985)	**+** ✓ Barely active (Lenny 1985)	**+** ✓ (Lenney 1985; Chen 1994)	**+** ✓ Kidney, Liver, Spleen, Lung, small intestine, … (Lenny et al. 1985)

0: Absent, +: little present, ++: moderately present, +++: abundantly present, ?: not investigated, 0/+: conflicting results or present in specific situations, 0/?: believed to be absent, but not certain. Abbreviations: CN1: serum carnosinase, CN2: tissue carnosinase, CNS: central nervous system.

Table 1B. The presence or absence of carnosine/anserine and the most important enzymes in the metabolism of carnosine in several tissues of **RODENTS.**

	Blood	Skeletal muscle	Brain/CNS	Other organs
Carnosine	**0/++** ✓ Fasting endogenous carnosine concentration: n.d. (Lee 2005) or very low (Aldini 2011) ✓ After supplementation (Aldini 2011) or after exercise (Nagai 2003)	**+++** ✓ (Zapp 1938; Davey CL 1960; Crush 1970)	**+++** ✓ Present in olfactory system (Ferriero 1975; Margolis 1977) ✓ But homocarnosine more prevalent (Abraham 1962; Ferriero 1975; del Rio 1977)	**+** ✓ Kidney (Peters, 2012, Riedl 2011, Kamal 2009), retina (Pfister 2011), Liver (Mong 2011), Spleen (Kamal 2009)
Anserine	**0/++** ✓ After supplementation (Kubomura 2010)	**+++** ✓ (Zapp 1938; Davey CL 1960; Crush 1970)	**0** ✓ (Bauer 1979; Biffo 1990)	**+** ✓ Kidney (Peters, 2012)
Carnosine synthase	**0**	**+++** ✓ mRNA expression (Miyaji 2012)	**++** ✓ mRNA expression (Miyaji 2012)	**+** ✓ mRNA detected in liver and kidney, but very low (Miyaji 2012)
CN1	**0** ✓ (Jackson 1991; Aldini 2011)	**0** ✓ (Teufel 2003)	**+** ✓ (Teufel 2003)	**+** ✓ Kidney (Margolis 1984; Teufel 2003; Peters 2011a)
CN2	**0** ✓ (Wood 1957)	**+** Barely active (Wood 1957; Kunze 1986)	**+++** ✓ (Kunze 1986)	**+** ✓ (Wood 1957; Kunze 1986)

0: Absent, +: little present, ++: moderately present, +++: abundantly present, 0/++: absent or n.d. but clearly present in certain situations, 0/?: believed to be absent, but not certain. CNS: central nervous system. Abbreviations: CN1: serum carnosinase, CN2: tissue carnosinase, CNS: central nervous system, n.d.: not detectable.

4. (BIO)CHEMICAL AND PHYSIOLOGICAL PROPERTIES OF CARNOSINE

'The ability of carnosine to prevent advanced glycoxidation end-products (AGEs) and advanced lipoxidation end-products (ALEs) formation, on the one hand, and the convincing evidence that these compounds act as pathogenetic factors, on the other hand, strongly support carnosine as a promising therapeutic agent for oxidative-based diseases, such as diabetes. The mechanisms by which carnosine inhibits AGEs and ALEs is still under investigation but an emerging hypothesis is that carnosine acts by deactivating the AGEs and ALEs precursors, and in particular the reactive carbonyl species (RCS) generated by both lipid and sugar oxidation.' (Vistoli 2012)

What are AGEs and ALES? When proteins (and in the case of ALEs it can be other macromolecules as well) are irreversibly and non-enzymatically modified by sugars and lipids (or their derivatives), then they are respectively called AGEs and ALEs.

Several studies have indicated that AGEs and ALEs have a *pathogenic role* in the development and progression of different oxidative-based diseases, such as diabetes (Baynes 2003; Yamagishi 2012; Yap 2012). Their biological (damaging) activity is related to different, and in some cases not yet well-defined, mechanisms. But they can for example 1) attack extracellular matrix proteins, such as collagen, resulting in crosslinking and a decrease of their elastic and mechanical functions (Verzijl 2002; Ciulla 2011), 2) modify plasma proteins (such as enzymes) and receptors on cells, resulting in disturbed normal function, 3) induce pro-inflammatory effects by activating signaling cascades through for example RAGE (receptor for AGEs)(Ramasamy 2005). Whether or not the damaging effect of ALEs includes such a receptor-mediated mechanism is not yet established.

As obvious from the above citation, the role of carnosine in the formation of AGEs and ALEs is multifactorial. This is a consequence of AGEs/ALEs being a very complex class of compounds that are formed by different mechanisms, by heterogeneous precursors and which can be formed both exogenously or endogenously. Although the mechanisms are very well studied *in vitro*, there is a huge gap in translating these studies into *in vivo* findings. Below, these mechanisms will be discussed (4.1. to 4.5) and an overview is provided in figure 4.

4.1. Anti-oxidant activity

Since the 80s, **Alexander A. Boldyrev** has dedicated his 'professional' life to study carnosine in relation to *oxidative stress,* leading to a book 'Carnosine and Oxidative Stress in Cells and Tissues' (Boldyrev 2006). Carnosine is supposed to exert its anti-oxidant effects by a direct and indirect mechanism.

In vitro evidence is provided that carnosine can **directly** inactivate reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) at physiological concentrations (Boldyrev 1988; Kohen 1988; Boldyrev 1992; Pavlov 1993; Klebanov 1997; Tamba 1998). ROS like $O_2^{\bullet -}$ and $OH^{\bullet}$ are both a cluster of two atoms that contain an unpaired electron (free radicals). They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can damage a broad range of macromolecules. It is assumed that the imidazole group is the preferential site for $O_2^{\bullet -}$ and $OH^{\bullet}$-attack (Tamba 1999).

When ROS are not neutralized, the most reactive hydroxyl radical ($OH^{\bullet}$) can further create *peroxyl radicals* ($ROO^{\bullet}$). The hydroxyl radical can easily act on the fatty acid side chains of lipids (in the various membranes of the cell, especially mitochondrial membranes), forming a lipid peroxyl radical, which can further evolve to reactive carbonyl species such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein. Here as well, the L-histidine moiety is required for the scavenging activity towards peroxyl radicals (Kohen 1988).

Caution is warranted, as Decker and coworkers re-evaluated the anti-oxidant activity of carnosine in 2000 because of the presence of contaminating hydrazine (ranging from 0.01 to 0.2%), a powerful reducing agent that can inactivate free radicals, in commercial carnosine preparations. They still confirm that purified carnosine was capable of scavenging lipid peroxyl radicals, although at a millimolar range (Decker 2000) and question the role of carnosine as a free radical scavenger in vivo. Importantly, following research indicated that the radical scavenging activity of purified carnosine is not due to reducing properties, meaning not acting as an electro-transfer agent (Aldini 2011).

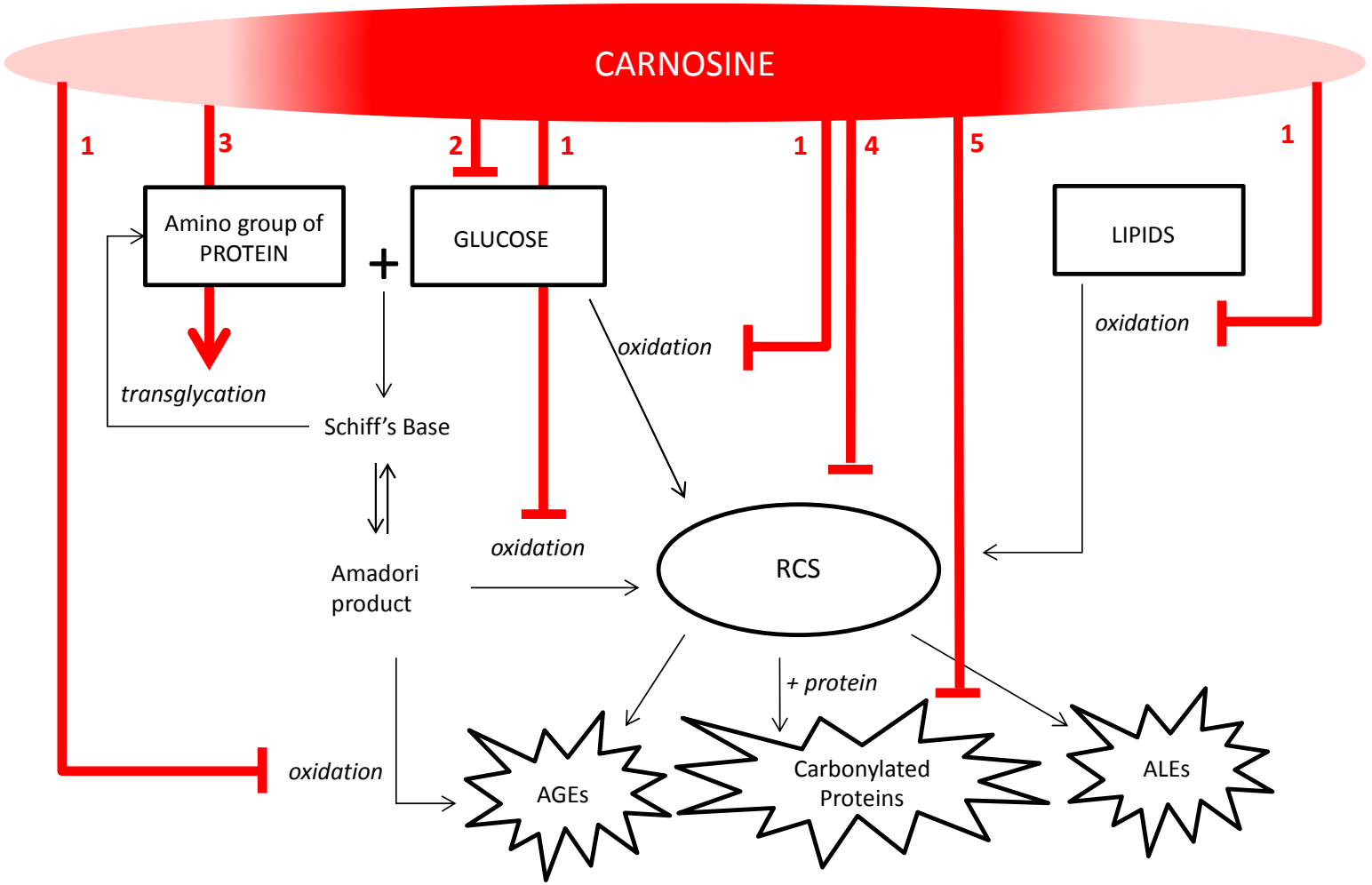

Figure 4. Carnosine can inhibit the formation of AGEs and ALEs by several proposed and evidence-based mechanisms: 1) an anti-oxidant mechanism, 2) a hypoglycemic mechanism, 3) a transglycating mechanism, 4) quenching of reactive carbonyl species (RCS), 5) quenching of carbonylated proteins. RCS: reactive carbonyl species, AGEs: Advanced glycation end-products, ALEs: advanced lipoxidation end-products.

Although the above literature points to no or limited role of carnosine as a direct anti-oxidant in vivo, carnosine does seem to be meaningful *in vivo* as an **indirect anti-oxidant**. Lee et al. (2005) were the first in demonstrating that carnosine improves the enzymatic anti-oxidant activity in streptozotocin (STZ)-induced diabetic Balb/cA mice. Carnosine supplementation (0.5 g/l or 1 g/l in drinking water) could restore decreased levels of catalase (CAT) and glutathione (GSH) peroxidase in diabetic liver and kidney (Lee 2005). In addition, aged rats receiving a daily dose of 250mg/kg (intraperitoneal) carnosine for one month, had less lipid peroxidation (malondialdehyde and diene conjugates) compared to aged control rats and depleted levels of blood GSH, superoxide dismutase (SOD) and glutathione peroxidase (GPX) were restored (Aydin 2010). This was even confirmed in young healthy rats, where supplemented rats had increased activities of enzymatic oxidants (SOD and GPX) in liver and skin and decreased lipid oxidation (MDA) compared to non-supplemented rats (Kim 2011). It appears that not only enzymatic anti-oxidants are 'spared' as Aydin et al. (2010) reported that vitamin E was significantly increased in carnosine-treated aged rats, pointing towards an non-enzymatic anti-oxidant mechanism as well. However, when rats were fed a vitamin E-deficient diet, carnosine supplementation could not increase antioxidant status of the rats (Maynard 2001; Ibrahim 2008).

Irrespective of carnosine showing anti-oxidant activities through a direct effect or through an indirect effect (enzymatic or non-enzymatic), it is demonstrated that carnosine indeed counteracts the formation of lipid peroxidation end-products, both in pathological (cfr infra) and exercise-induced conditions (Boldyrev 1988; Dawson, Jr. 2002). For example, Dawson et al. (2002) demonstrated that downhill running for 90 minutes increased TBARS (thiobarbituric acid reactive substances) levels in the extensor digitorium longus (EDL) muscle in rats and this was completely blunted by a supplementation-induced increase in muscle HCD.

Conclusion: The anti-oxidant effect of carnosine is related to a direct and indirect effect. The direct effect points to the scavenging of ROS, but although this is extensively established *in vitro*, it has a very limited role *in vivo*. The indirect effect is related to the increase or sparing of enzymatic and/or non-enzymatic anti-oxidants and is confirmed in animal models *in vivo*.

4.2. An anti-glycator and hypoglycemic agent

Alan Hipkiss, the discoverer of carnosine 'as an anti-glycating agent', started his work by demonstrating that carnosine has the ability to form adducts with reducing sugars like glucose (Hipkiss 1994; Hipkiss 1995). In this light, carnosine would be already active in the first step of AGE formation, i.e. preventing that the carbonyl group of reducing sugars is reacting with the amino group of lysine, by serving as an alternative and competitive glycation target (number 2 in figure 4). The anti-glycating property of carnosine has been confirmed by several other *in vitro* studies (Seidler 2000; Yan 2005; Pepper 2010). Of course, it can be questioned whether this is relevant *in vivo* as carnosine (if present in circulation) will not be able to quench the huge amount of glucose in the blood in a diabetic state.

In 2005, Szwergold and coworkers documented another mechanism by which carnosine and anserine can retard or reverse glycation, i.e. *transglycation*. The process involves decomposition of the very first intermediates of the non-enzymatic glycation cascade (aldosamines a.k.a. Schiff bases) by nucleophilic attack of carnosine and/or anserine on the preformed aldosamine such as glucosyl-lysine. As consequence, glucosyl-carnosine or anserine is formed instead of glucosyl-lysine (Seidler 2004; Szwergold 2005) (pathway number 3 in figure 4).

A completely different mechanism by which carnosine affects blood glucose is by acting on plasma insulin (hormone that decreases blood glucose). Nagai and colleagues believe that carnosine has hypoglycemic effects through the regulation of the autonomic nervous system via the histamine H3 receptor (Yamano 2001; Nagai 2003; Kubomura 2010). In a first study, L-carnosine was injected into the intraperitoneal space, the right lateral cerebral ventricle (LCV) or the stomach (Yamano 2001). Immediately after L-carnosine administration 2-deoxy-D-glucose was injected into the LCV. Nerve filaments were isolated from central cut ends of autonomic adrenal, hepatic and pancreatic nerves to record efferent activities with a pair of silver electrodes. Central or peripheral administration of specific doses of L-carnosine reduced the 2DG-hyperglycemia, inhibited neural activities of sympathetic efferent nerves innervating the adrenal gland and liver and facilitated the activity of vagal celiac nerve innervating the pancreas. In addition,

plasma insulin was increased and plasma glucagon decreased. These results were confirmed by two following studies with different protocols, here STZ-induced diabetic rats were used and oral doses of carnosine were given (0.1g, 0.01g and 0.001g L-carnosine was mixed in 100g control diet for 1 week) (Nagai 2003) or anserine was used in stead of carnosine (Kubomura 2010). The latter study is of great importance as they also supplemented anserine in five healthy humans at the start of an oral glucose tolerance test. Anserine ingestion (10 and 100 mg/45 kg body weight) tended to reduce blood glucose levels during the tolerance test compared with ingestion of tap water only ($0.05<p<0.10$).

In line with the aforementioned, Sauerhofer et al. (2007) reported also increased fasting serum insulin levels in L-carnosine-supplemented db/db mice compared to non-supplemented db/db mice. A later and milder manifestation of diabetes was observed in the supplemented mice, together with enlarged pancreatic islets containing predominantly insulin-producing β-cells.

Conclusion: Animal studies demonstrate that carnosine has hypo-glycemic effects, which is possibly mainly related to the ability to increase plasma insulin. This work will study the effect of chronic carnosine supplementation on a glucose tolerance test in high-fat diet rats.

4.3. Quencher of reactive carbonyl species

Reactive carbonyl species (RCS) are a class of compounds having a carbonyl group () and which behave very reactive towards the nucleophilic residues of biomacromolecules (protein, DNA and RNA). In literature, RCS refer predominantly towards reactive low-molecular weight or short chain aldehydes () such as α,β- unsaturated aldehydes (e.g. HNE and acrolein), di-aldehydes (e.g. MDA, glyoxal,…) and keto-aldehydes (e.g. methylglyoxal), however strictly speaking they also refer to reducing sugars as they possess an aldehyde group as well *or* to the subsequently formed protein carbonyls as a carbonyl group is introduced into proteins *or* to many other carbonyl compounds arising from sugars and lipids. Nevertheless, in this work, the term RCS will refer to the first mentioned group (as most often used in literature (figure 5) (Aldini 2005; Vistoli 2013).

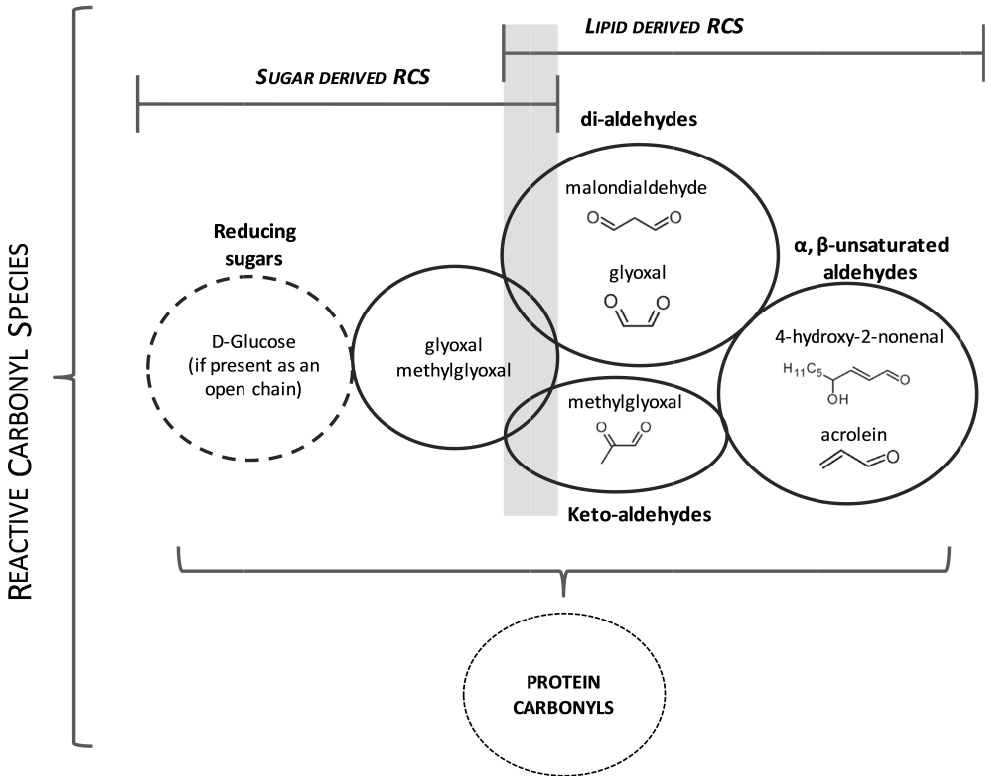

Figure 5. Schematic presentation of reactive carbonyl species (RCS) derived from sugar and lipid metabolism. Circles in dotted lines are also sometimes classified as RCS, but in this work, RCS will refer to the species mentioned in the full circles.

Reactive aldehydes are mainly produced by lipid peroxidation and the lipid metabolism. The most investigated RCS is HNE. It is one of the most abundant and lipid-derived RCS and it can be generated endogenously from PUFAs, present in membrane phospholipids (Catala 2009). It should, however, be stressed that some RCS such as glyoxal and methylglyoxal (smallest di-aldehydes) are generated by both lipids and sugars (figure 5)(Vistoli 2013).

Several *in vitro* studies have demonstrated that carnosine and its methylated form anserine can react with α,β-monounsaturated aldehydes, such as HNE and acrolein (number 4 in figure 4) (Zhou 1999; Aldini 2002a; Aldini 2002b; Liu 2003; Carini 2003; Orioli 2005). Figure 6 demonstrates the chemical reaction of carnosine with the cytotoxic HNE. Carnosine was 10 times more active as an HNE quencher than L-histidine while beta-alanine was totally inactive, which points to the fact that the two constitutive amino acids act synergistically when incorporated as a dipeptide. In addition, from figure 6 it is clear that beta-alanine moiety catalyzes the addition reaction of the histidine moiety to HNE.

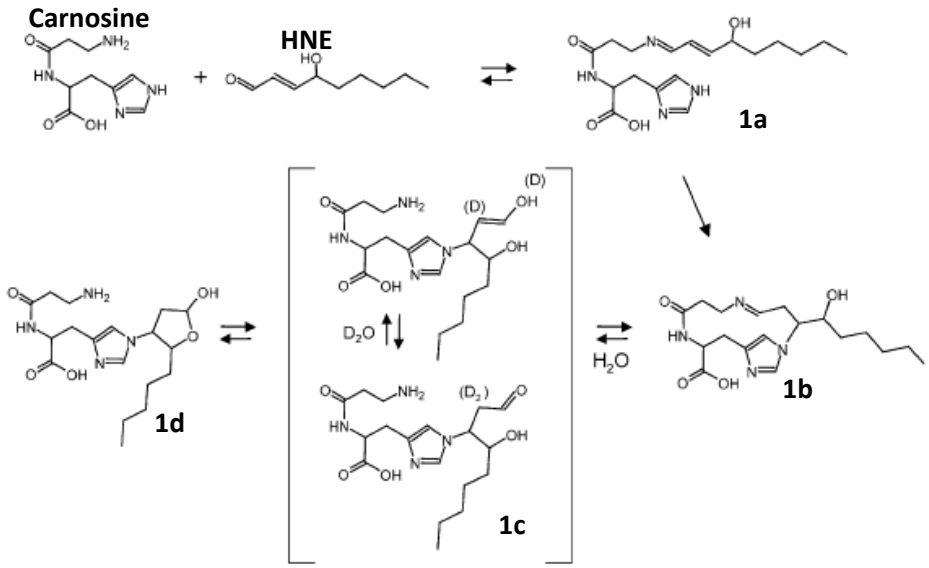

Figure 6. The reaction of carnosine with HNE (4-hydroxy-2-nonenal). The reaction is initiated by the beta-alanine moiety, forming the reversible α,β unsaturated imine 1a (Aldini 2002b).

The biological role of carnosine as a quencher of α,β-unsaturated aldehydes was then verified by detecting carnosine-HNE reaction adducts in oxidized rat skeletal muscle homogenate. Anserine, the N_{π}-methyl derivative of carnosine, showed quenching activity similar to carnosine, suggesting that the N_{τ}-atom of carnosine is probably involved in the HNE quenching reaction. In imitation of HNE, a similar reaction was then elucidated for acrolein by the same lab (Carini 2003).

To date, there is only one paper available investigating carnosine-HNE adducts *in vivo*. Aldini et al. (2011) reported higher levels of carnosine-HNE adducts in urine of Zucker obese rats compared to the lean control animals, which confirmed HNE overproduction in obese animals and the role of carnosine as an endogenous detoxifying agent of RCS.

Conclusion: Strong *in vitro* evidence exists that carnosine can act as a scavenger of reactive carbonyl species. However, only 1 study has studied and detected carnosine-HNE adducts in urine of Zucker obese rats. This work will examine the presence of these adducts in a high-fat diet rat model in order to assess the scavenging characteristics of carnosine *in vivo*.

4.4. Quencher of protein carbonyls

Protein carbonyls are partly formed by reactive carbonyl species or its derivatives (c and d in figure 7), but they can also be formed through direct oxidation of proline, arginine, lysine and threonine residues or by oxidative cleavage of the protein backbone. These major pathways are represented in figure 7.

Hipkiss and coworkers well demonstrated (although in vitro) that carnosine is not only able to prevent protein carbonylation (which is a logical consequence of carnosine being an inhibitor and quencher of RCS, cfr supra), but it can also directly react with protein carbonyl groups, forming protein-carbonyl-carnosine adducts. These adducts are given the name 'carnosinylated proteins' (pathway number 5 in figure 4) (Brownson 2000; Hipkiss 2000b; Hipkiss 2001; Hipkiss 2002). However, up to now, no evidence of such protein-carbonyl-adducts, existing *in vivo,* is presented.

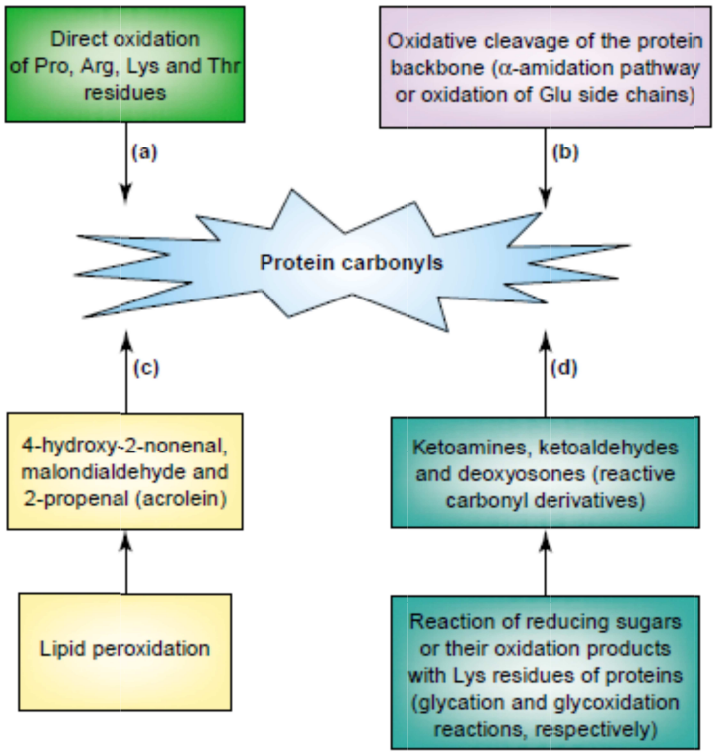

Figure 7. The four major pathways in the formation of protein carbonyls are represented (Dalle-Donne 2003).

Nevertheless, *in vivo* evidence exists that carnosine can decrease the presence of protein carbonyls. Nagasawa et al. (2001) demonstrated that muscle carnosine loading in Sprague-Dawley rats significantly blunted the increase in muscle protein carbonyl content, induced by an intraperitoneal injection of Fe-nitrilotriacetate (Nagasawa 2001). In addition, Aldini et al. (2011) showed that increased kidney protein carbonyls in Zucker Obese rats (compared to the lean counterparts) were significantly decreased if rats received 30mg carnosine/kg body weight in their drinking water for 24 weeks.

Conclusion: Carnosine can decrease protein carbonyl levels *in vivo*, however, evidence of the ability of carnosine to *directly* quench protein carbonyls is currently lacking.

4.5. Inhibitor of ALEs and AGEs

'AGEs (advanced glycation end-products) represent a class of modified proteins, involving sugars and their degradation products and are generated by oxidative and non-oxidative pathways. The term ALEs (advanced lipoxidation end-products) includes a variety of covalent adducts, which are generated by the non-enzymatic reaction of RCS (derived from lipids) with the nucleophilic sites of macromolecules such as proteins, DNA and aminophospholipids.' (Vistoli 2013)

When looking into the formation mechanisms, ALEs and AGEs are not so entirely different as it seems from a first point of view. Both classes have non-enzymatic covalently modified proteins and oxidative stress is an important player in their formation (although not always). Moreover, the name EAGLEs (Either Advanced Glycation or Lipoxidation End products) has been created since adducts can be both AGEs and ALEs (Vistoli 2013). For example, carboxymethyllysine (CML) is the most dominant AGE, but is an ALE as well. It is generated by glyoxal, which can be derived from both lipid and sugar oxidative degradation pathways (Fu 1996).

As consequence of the aforementioned properties of carnosine (chapters 4.1. to 4.5.), this dipeptide should have an inhibitory effect on the formation of AGEs and ALEs (figure 4). Only three recent papers are available studying the effect of carnosine on AGEs/ALEs *in vivo*. Aldini et al. (2011) demonstrated that (3-fold) increased urinary AGEs in Zucker obese rats (compared to the lean counterparts) were reduced with 50% if Zucker obese

rats were supplemented with L-carnosine for 24 weeks. On the other hand, two recent studies demonstrated that, although carnosine supplementation was effective in protecting retinal capillary cells and kidneys from apoptosis in STZ induced diabetic retinopathy/nephropathy, carnosine could not reduce CML and methylglyoxal formation in the respective tissues (Pfister 2011; Riedl 2011).

Conclusion: It seems very likely that carnosine can inhibit the formation of AGEs and ALEs in vivo, as carnosine can act on AGEs and ALEs precursors. However, only 3 studies have looked into this association and they are contradictive. This work will examine the effect of carnosine on CML levels (an important EAGLE) in both blood and urine of high-fat diet rats.

4.6. Other characteristics

This chapter will briefly discuss two other recently reported characteristics of carnosine that are of interest for a diabetic or obese population. Finally, two sports-related characteristics will be mentioned, because of their world-wide impact in the performance enhancement field.

4.6.1. **Anti-inflammatory and lipid-lowering**

Lee et al. (2005) provided the first evidence of carnosine having *anti-inflammatory effects.* Balb/cA mice were made diabetic by STZ-treatment. The levels of serum interleukin (IL)-6 and tumor necrosis factor (TNF)-α, two pro-inflammatory cytokines, were significantly increased in diabetic mice. The intake of carnosine and histidine (both 0.5 and 1 g/l) showed dose dependent suppressive effects in the release of IL-6, but only the higher dose (1g/l) significantly reduced TNF-α level. No differences were reported between carnosine and L-histidine treatment.

The anti-inflammatory effects were later confirmed by Liu and coworkers (2008). Ethanol treatment caused up-regulation of both IL-6 and TNF-a mRNA expression in liver and elevated levels of c-reactive protein (CRP), IL-6 and TNF-α in liver. The intake of histidine and carnosine significantly and dose-dependently down-regulated mRNA expression of IL-6 and TNF-α and diminished the release of CRP, IL-6, and TNF-alpha (Liu 2008). Finally, the anti-inflammatory protection from carnosine was also confirmed in the striatum of MPTP-

treated mice (Tsai 2010). MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, is widely used to induce Parkinson disease, a neurodegenerative disease by which oxidative stress and neuro-inflammatory processes have been implicated as important mechanisms responsible for neuronal death.

The prominent study of Lee et al. (2005) demonstrated also a *lipid lowering effect*, as elevated triglyceride and cholesterol levels in heart and liver of (STZ-induced) diabetic Balb/cA mice were significantly reduced (and almost restored) after carnosine supplementation. Later animal studies could confirm beneficial effects on dyslipidemia in both plasma (Aldini 2011) and tissues (Mong 2011).

However, whether carnosine has a *direct* anti-inflammatory and lipid-lowering effect is uncertain and cause-effect relationships are unclear. Indeed, it is possible that the protective effects are a consequence of decreased systemic and tissue stress (lowered oxidative stress and better glycemic control in the study of Lee et al. (2005)).

Conclusion: Carnosine has anti-inflammatory and lipid-lowering characteristics, demonstrated by animal studies. This work will investigate the possible role of carnosine as an anti-inflammatory and lipid-lowering agent in both rodents and humans.

4.6.2. **Improving contractile function of skeletal muscle**

Improving contractile function of the muscle is the very first discovered characteristic of carnosine. Already in 1953, Severin and colleagues (pupil of carnosine's discoverer Gulewich) provided the first evidence for the capacity of carnosine to attenuate peripheral fatigue in frog sartorius muscle (Severin 1953). More than hundred publications and more than 50 years later, several reviews and meta-analysis provide relative good evidence that possessing high muscle carnosine content is advantageous for high-intensity exercise capacity (Derave 2010; Baguet 2010; Hobson 2012; Derave 2012; Sale 2013). More specifically, Hobson et al. (2012) concluded that beta-alanine supplementation, which was already been shown to be the rate-limiting precursor of muscle carnosine loading, is beneficial for exercise types of 1 to 4 minutes. The effect was less pronounced (=0.046) in exercise types lasting longer than 4 minutes, and was not significant for shorter duration exercise (< 1min).

The performance enhancing effect is partly related to the protection against exercise-induced-acidosis (pH buffering). Carnosine has a pKa value of 6.83 (which is similar for anserine and 6.1 for L-histidine), whereas pH level in muscle at rest is 7.05 and can decreases up to ~6.4 because of intense exercise. Knowing that the capacity of a metabolite to accept protons is maximal when its pKa value equals the pH of the surrounding medium, suggests that carnosine is a good candidate to be an important intracellular buffer (Bate-Smith 1938; Davey CL 1960). In this light, one of the 2 nitrogens of the imidazole ring of carnosine can be protonated in the physiological pH range (Abe 2000).

A second proposed ergogenic mechanism of carnosine is improving *calcium handling* in skeletal muscle. Muscle contraction occurs when cross bridges between actin and myosin can be formed. It is a complexly regulated chain of events, but one of the first steps is the release of Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} will then bind to troponin, which will cause a conformational change necessary to allow cross-bridge formation between actin and myosin. Carnosine has been suggested to counteract the declined, exercise-induced, Ca^{2+} availability by two different mechanisms. First, Russian studies (Batrukova 1997; Rubtsov 2001) on skinned muscle fibers showed that carnosine increases Ca^{2+} release from the sarcoplasmic reticulum. However, these conclusions were criticized by others (Dutka 2004) as non-physiological settings were applied. A second mechanism by which carnosine can counteract the decreased Ca^{2+} availability was proposed by Dutka and Lamb (2004). Experiments on rat (Dutka 2004) and human (Dutka 2012) skinned fibers showed that for a given concentration of Ca^{2+} in the sarcoplasm, the produced force was higher in the presence of carnosine and this points to increased Ca^{2+} sensitivity instead of an increase in calcium release.

5. THE CARNOSINE-CARNOSINASE SYSTEM IN A DIABETIC STATE

5.1. Diabetes and its insurmountable metabolic changes

Diabetes Mellitus is a collective term for a condition in which glucose builds up to abnormal levels in the bloodstream, causing serious health problems. In general, the ability to maintain normal blood glucose levels involves a complex interplay between insulin secretion by pancreatic beta-cells and insulin respons in tissues such as liver, adipose tissue and skeletal muscle.

The two most common forms of diabetes are diabetes mellitus type 1 and type 2. Diabetes mellitus type 1 (also known as the formerly insulin dependent diabetes or juvenile diabetes) is a form of diabetes that results from auto-immune destruction of insulin-producing beta-cells of the pancreas and is believed of having a large genetic component. On the other hand, diabetes mellitus type 2 (formerly non-insulin dependent diabetes mellitus or adult-onset diabetes) has a metabolic component and is often linked to obesity and a sedentary lifestyle. It accounts for 90% of all cases of diabetes and it is characterized by the combination of decreased insulin secretion and decreased insulin sensitivity. However, many other less common/known forms of diabetes exist, such as monogenetic forms like MODY 'Maturity Onset Diabetes of the Young'-variants.

Although type 1 and type 2 diabetes have long been viewed as two different diseases, researchers, however, are currently showing that both types have more in common than once believed. For example, both forms are characterized by progressive destruction of pancreatic beta-cells via a cytokine-induced apoptosis. In type 2 diabetes mellitus, apoptosis is progressively favored mainly by glucotoxicity and lipotoxicity; whereas in type 1 diabetes mellitus, apoptosis is rapidly induced by irreversible autoimmune process (Cnop 2005; Daneman 2006).

In addition, both type 1 and type 2 diabetes lead to similar **long-term complications** and metabolic problems. Large prospective clinical studies show a strong relationship between glycaemia and diabetic microvascular complications (retino-, nephro- and neuropathy) in both type 1 and type 2 diabetes (The Diabetes Control and Complications Trial Research Group 1993; UK Prospective Diabetes Study (UKPDS) Group 1998).

Furthermore, hyperglycemia and insulin resistance (both independently) seem to have important roles in the pathogenesis of macrovascular complications (atherosclerosis and cardiovascular disease such as stroke, ischemic disease,…)(Wei 1998; Ebara 2000; Ginsberg 2000).

The last decades, four main *metabolic pathways/disturbances* have been put forward for being responsible for diabetic microvascular and macrovascular complications (figure 8):

1) increased polyol pathway flux (discovered in 1966):
   This leads to decreases in NADPH, which is an essential cofactor for regenerating reduced glutathione (GSH), a critical intracellular anti-oxidant. By decreasing GSH, the polyol pathway increases the susceptibility to intracellular oxidative stress. Second, increased flux through the polyol pathway increases NADH:NAD+ ratio, which inhibits GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and thus increases triose phosphate, leading to elevated levels of AGEs and protein kinase C.
2) increased hexosamine pathway flux (discovered in late 1990s):
   N-acetyl glucosamine influences transcription factors such as an increase in plasminogen activator inhibitor-1 (PAI-1), responsible for vascular inflammation and thrombosis.
3) activation of protein kinase C (PKC) β- and δ-isoforms (discovered in late 1980s):
   induces blood flow abnormalities, vascular permeability, capillary occlusion, pro-inflammatory gene expression and increases ROS production.
4) and increased AGE formation (discovered in late 1970s):
   damaging effect is explained in chapter 4.5.

We will not explore these pathways into further detail, but it is clear that diabetes has several insurmountable metabolic changes.

Until 2001, there was no unifying mechanism linking these four mechanisms. Brownlee M. (2001 and 2005) discovered that ***the overproduction of superoxide by the mitochondrial electron transport chain*** unifies the four well-known pathways (figure 8). This was published in *an insight review in NATURE* (Brownlee 2001) and in *The Banting Lecture 2004* (Brownlee 2005). Briefly, in diabetic cells with high glucose levels (especially non-insulin dependent cells, such as retinal cells, glomerulus, nerves), there is too much glucose being oxidized in the citric acid cycle, ultimately leading to overproduction of

superoxide. Exactly this overproduction of superoxide activates the four damaging pathways by inhibiting GAPDH (glyceraldehyde-3phosphate dehydrogenase) (START, figure 8). When GAPDH activity is inhibited, the level of all the glycolytic intermediates that are upstream of GAPDH increase, such as glyceraldehyde-3-phosphate (activating pathway 3 and 4), fructose-6 phosphate (activating pathway 2) and glucose (activating pathway 1).

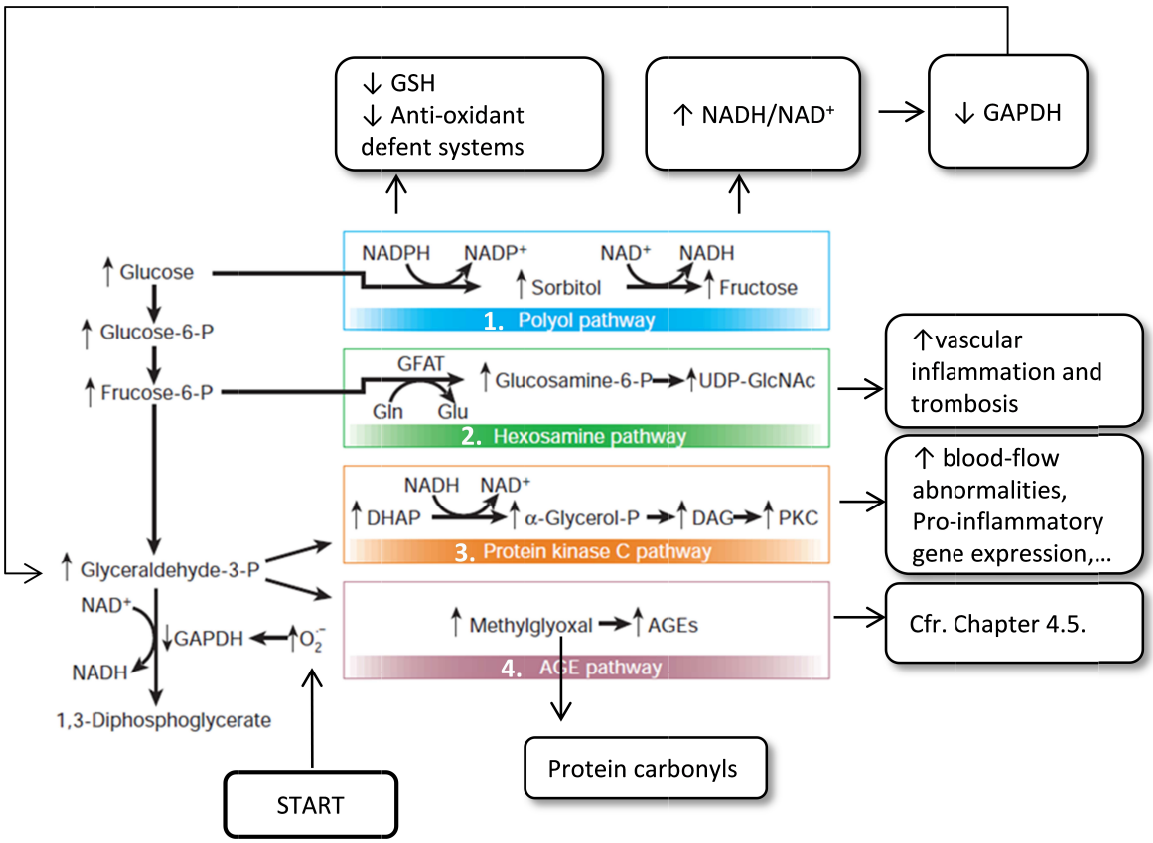

Figure 8. Representation of the four main pathways by which hyperglycemia can cause diabetic complications. The unifying mechanism linking these pathways is hyperglycemia-induced mitochondrial superoxide overproduction (START) (Brownlee 2001).

But how does the unifying mechanism explains diabetic macrovascular disease? In contrast to diabetic microvascular disease, data from the UKPDS has shown that hyperglycemia is not the major determinant of diabetic macrovascular disease. Therefore, The San Antonio Heart Study was set up to discriminate between increased macrovascular risk due to insulin resistance (and its associated abnormalities, such as disturbed lipid profile and blood pressure) and increased risk due to hyperglycemia. The results were clear, high insulin resistance increases cardiovascular risk by 2.5-fold, moreover, after adjustment for 11 known cardiovascular risk factors such as low-density

lipoproteins (LDL), high-density lipoprotein (HDL), triglycerides, blood pressure, smoking,… the insulin–resistant subjects had still a 2-fold increase of cardiovascular disease. So, insulin resistance is the major player in macrovascular disease. Coming to our point, insulin resistance causes increased free fatty acid (FFA) flux from adipocytes into macrovascular (arterial), not in microvascular, endothelial cells, resulting in increased FFA oxidation by the mitochondria. This generates on his turn the same electron donors (NADH en FADH2) produced by glucose oxidation, resulting in mitochondrial overproduction of ROS by exactly the same mechanism as for hyperglycemia.

This work will focus on diabetes type 2 and the first stage of the disease, namely **insulin resistance**. Insulin resistance (or also called prediabetes type 2) can develop as a result of unhealthy lifestyle (overeating and physical inactivity) and genetic predisposition (Shigeta 2001; Wybranska 2007). It is still under debate which organ (skeletal muscle, liver or adipose tissue) is the first target of insulin resistance and which organ can be accounted for being the primary defect in the development of type 2 diabetes (Kraegen 1991; Frayn 2001; DeFronzo 2009), but it cannot be denied that the **skeletal muscle plays an important role.** It comprises ~40% of body mass and mediates over 75% of all insulin-mediated glucose disposal under normal physiological circumstances, however it decreases significantly in insulin resistant individuals (DeFronzo 1985; Stump 2006; Abdul-Ghani 2010). Several theories about the development of skeletal muscle insulin resistance are postulated. First, a body of research proposes that excess delivery of fatty acids to the skeletal muscle results in the accumulation of intramyocellular lipid metabolites such as diacylglycerol and ceramides, which are both detrimental for the insulin signaling pathway (Kraegen 2008; Martins 2012). Second, excess fatty acid oxidation in the mitochondria charges the electron transport chain, resulting in an overproduction of ROS and subsequently lipid and protein oxidation will occur (Wei 2008; Kewalramani 2010). Lipid oxidation products such as HNE can inhibit insulin-induced phosphorylation of PKB/Akt (necessary for an adequate insulin signaling) (Pillon 2012). Ingram et al. (2012) demonstrated a negative correlation between protein-HNE levels in skeletal muscle and glucose disposal rate (by an hyperinsulinemic-euglycemic clamp) in prediabetic and diabetes type 2 patients (Ingram 2012). Finally, emerging data indicate that the excessive activation of inflammatory pathways may represent a fundamental step in the

development of insulin resistance (Wei 2008; Kewalramani 2010). For example, mice lacking TNFα (or its receptor) or iNOS are protected from obesity-induced skeletal muscle insulin resistance (Uysal 1997; Perreault 2001).

Conclusion: Skeletal muscle insulin resistance is one of the first signs of diabetes type 2. Once diabetes is present, hyperglycemia and oxidative stress go hand in hand to induce metabolic changes (increased polyol pathway flux, increased AGE formation, activation of PKC, increased hexosamine pathway flux), leading to micro- and macrovascular complications.

5.2. Carnosine and diabetes

Taken together the chapters *'biochemical and physiological properties of carnosine'* and '*diabetes and its insurmountable metabolic problems'*, it seems like the molecule carnosine is a very good candidate for being protective in diabetes and its complications. A schematical representation of the intersection between these two chapters is represented in figure 9.

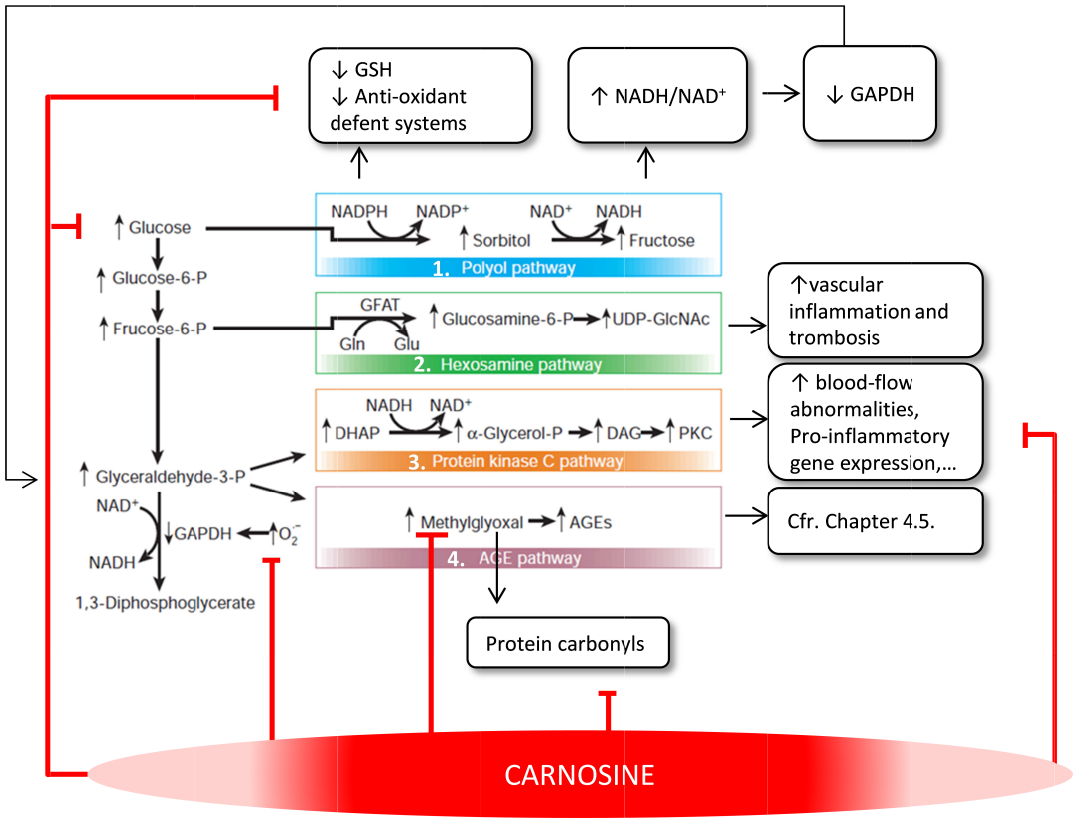

Figure 9. A schematic representation of the intersection between the chapters *'biochemical and physiological properties of carnosine'* and '*diabetes and its insurmountable metabolic problems'*.

Briefly, the diabetes-related metabolic problems, possibly influenced by carnosine, are: increased reactive carbonyl species (methylglyoxal, HNE), protein carbonylation (as a consequence of increased RCS), formation of AGEs, a weakened anti-oxidant defense system and increased low-grade inflammation. We can conclude that carnosine possesses properties to prevent diabetes-related complications, but more importantly it can be expected that carnosine can already act on mechanisms responsible for insulin resistance (the initial phase of diabetes type 2). .

The combination of *carnosine* being predominantly and abundantly present in the skeletal muscle and the *skeletal muscle* being an important 'organ' in the development of insulin resistance, rises the hypotheses that increasing muscle carnosine content can be protective in the development of insulin resistance. To recapitulate, *if* carnosine indeed acts as a lipid lowering agent (and thus decreasing free fatty acid flux towards skeletal muscle), increases the anti-oxidant defense system inside the muscle, quenches reactive carbonyl species such as HNE and serves as an anti-inflammatory agent, *then* the insulin signaling pathway will be protected by increasing muscle carnosine stores (as these factors are known to directly and indirectly influence the insulin signaling pathway and thus glucose uptake in skeletal muscle). Off course, caution is warranted as most mechanisms are based on *in vitro* studies.

To fully explore the role of carnosine in a diabetic setting **two approaches** must be followed. **First**, it must be investigated how the carnosine-system behaves in a diabetic setting, by looking at carnosine and carnosinase concentrations in respectively tissues and circulation. **Second**, the additional effect of artificially increased plasma or tissue carnosine must be explored in a diabetic/insulin resistant setting.

Concerning the latter, table 2 provides an overview of all in vivo studies done with carnosine supplementation in diabetic rodent models focusing on the protective effect of carnosine. From this table it is clear that up to now, predominantly severe diabetic models are used (i.e. genetically predetermined, STZ-induced diabetes or hyperglycemia induced by 2DG-injection of LCV) and no attention has been given to the role of the skeletal muscle (the predominant synthesis location of carnosine and an important organ

Table 2. An overview of all *in vivo* studies with carnosine supplementation in diabetic rodent models.

Study	Species	Administration protocol	Duration	Effect
(Yamano 2001)	Wistar rats: Hyperglycemia induced by LCV-injection of 2DG	Chicken Essence (containing L-CAR)	1 week	↓ 2DG-hyperglycemia
		IP injection of L-CARN (5 µmol to 5 nmol)	acute	↓ blood glucose (0.05 and 0.5 nmol), ↑ plasma insulin, ↓ plasma glucagon, ↓sympathetic efferents of adrenal and liver, ↑parasympathetic efferents of pancreas (0.5 nmol).
(Nagai 2003)	Wistar rats: Hyperglycemia induced by daily LCV-injection of 2DG	0.001%, 0.01% and 0.1% (diet)	1 week	↓ hyperglycemia (0.01% and 0.001%)
(Lee 2005)	STZ-induced diabetic Balb/cA mice	L-CAR: 0.5 and 1 g/l (water) HIST: 0.5 and 1 g/l (water)	4 weeks	↓ hyperglycemia, ↓ hyperlipidemia in heart and liver, ↑enzymatic anti-oxidant capacity,↓ inflammation in plasma (equal effects for histidine and carnosine) [carn] heart and liver: = , [hist] in plasma, heart and liver ↓ vs CON
(Sauerhofer 2007)	db/db mice	L-CAR: 4mM (~0.1%,~1g/l water)	17 weeks	↓ fasting glucose, ↑ fasting insulin, enlarged pancreatic islets (+ effect on development of diabetes type 2) No effect on diabetic nephropathy
(Soliman 2007)	STZ-induced diabetic albino rats	L-CAR: 100 or 200 mg/kg BW/day (IM)	4 weeks (2 weeks before and 2 weeks after STZ)	↓ hyperglycemia, ↓ hyperlipidemia ↑liver function and↑ vitamin E status.
(Yan 2008)	STZ-induced diabetic rats	L-CAR: 5 or 10 mg/ml in eye drops, 2x/day	14 weeks	↓ formation of AGEs, ↓ progression of lens opacification (only in early-stage cataract)

(Kamei 2008)	STZ-induced diabetic mice	Zinc L-CAR (1:1 at MW): 75-300 mg/kg p.o.	8 weeks	ameliorates abnormal sensory perception
(Mong 2011)	C57BL/6 mice receiving 60% high-fat diet	L-CAR: 1 g/l (water) HIS: 1 g/l (water)	8 weeks	↓ lipogenic enzymes and transcription factors for fatty acid and cholesterol metabolism (SREBPs) ↓ body weight, epididymal and hepatic fat ↓ hyperinsulinemia and insulin resistance [carn] and [hist] in liver ↓ in HF compared to CON
(Aldini 2011)	Zucker Obese Rats	L-CAR: 30mg/kg BW/day (water) D-CAR: 30mg/kg BW/day (water)	24 weeks	↓ hyperlipidemia, ↓ hypertension, ↓ renal injury, ↓ protein carbonylation, ↓ AGEs and RCS [carn]plasma and liver: seems to be ↓(n.s.) in Zucker Obese vs lean
(Pfister 2011)	STZ-induced diabetic rats	L-CAR: 1 g/kg BW/day (water)	6 months	↓ development diabetic retinopathy, protects retinal capillary cells [carn]retinal cells ↓ in diabetic rats vs healthy rats
(Riedl 2011)	STZ-induced diabetic rats	L-CAR: 1 g/kg BW/day (water)	3 months	↓ development diabetic nephropathy (prevents glomerular apoptosis and podocyte loss) [carn]glomerular cells ↓ in diabetic rats vs healthy rats

Abbreviations: LCV: lateral cerebral ventricle, 2DG: 2-deoxy-D-glucose, STZ: streptozotocin, L-CAR: L-carnosine, HIS: L-histidine, HF: high-fat, CON: control, AGEs: Advanced glycation end-products, RCS: reactive carbonyl species, p.o.: per os, IP: intraperitoneal, IM: intramuscular, n.s.: not significant, ↓: decreases/inhibits, ↑: increases/promotes.

in the development of insulin resistance). In humans, there are no supplementation studies in diabetic populations.

Concerning the first approach (endogenous carnosine system in a diabetic setting), Hipkiss and coworkers already speculated a decade ago that carnosine concentrations can be possibly decreased in tissues because of its characteristic of being a quencher of several deleterious species (oxidants, reactive carbonyl species, protein carbonyls….) (Brownson 2000; Hipkiss 2000b; Hipkiss 2001; Hipkiss 2002). His theory about the various fates of carnosinylated proteins is represented in figure 10. However, it must be stressed that this hypothesis is based on chemical experiments only, and no evidence for its involvement in biological events is available at present.

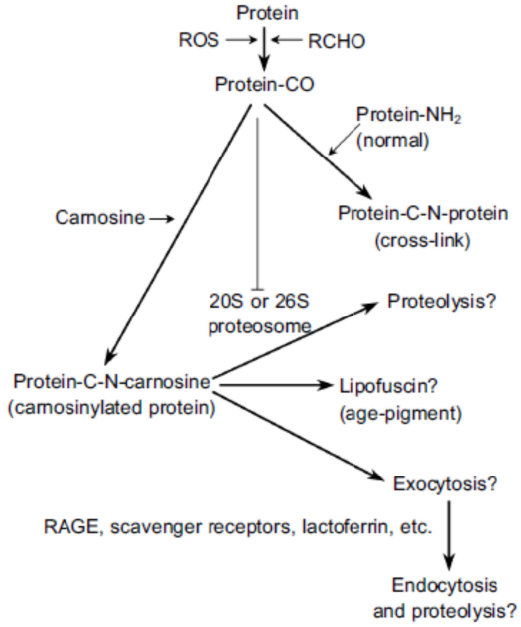

Figure 10: Possible fates of carnosinylated proteins. Abbreviations: ROS: reactive oxygen species, RCHO: reactive aldehyde or reducing sugar, Protein-CO: protein-carbonyl, RAGE: receptor for advanced glycation end-products (hipkiss 2000a).

Nevertheless, it has recently been demonstrated that carnosine concentrations are indeed decreased in retinal and glomerular cells in STZ- induced diabetic rats with respectively retinopathy and nephropathy (Pfister 2011; Riedl 2011). In addition, this decrease appears to happen in diabetic liver as well (Mong 2011), although not always significant (Aldini 2011). For skeletal muscle, contradiction exists. In rodents, only one paper is available demonstrating decreased carnosine levels in diaphragm skeletal muscle

of STZ-induced diabetic rats (Buse 1980). In humans, only two papers exist and they are contradictive. Gualano et al. (2012) reported reduced gastrocnemius carnosine content (-45%) in type 2 diabetic patients (Glycated hemoglobin 'HbA1c': 7.5 ± 0.3%; age: 60 ± 6 years), but not in soleus muscle, nor in type 1 diabetic patients (Gualano 2012). On the other hand, Srikanthan et al. (2012) demonstrated an increase in soleus carnosine in 10 diabetic type 2 patients (age: 54 ± 13 yrs; HbA1c: 6.0 ± 0.5%; patients on oral and insulin therapy were excluded (Srikanthan 2012).

Conclusion: Carnosine is a likely candidate to counteract the development of diabetes and diabetes-related complications because of its properties as an anti-oxidant, an anti-glycator, an anti-inflammatory and a lipid-lowering agent, a quencher of reactive carbonyl species, an inhibitor of protein carbonylation and AGEs/ALEs. There are some indications that carnosine content in tissues is possibly decreased in a diabetic state, however contradiction exists. In addition, carnosine supplementation studies are limited to severe diabetic rodents models and no attention was given to the skeletal muscle. Therefore, this work will 1) examine muscle carnosine concentrations in rodents and human with various degrees of glucose intolerance and 2) investigate the protective effect of carnosine supplementation in a very mild obese-insulin resistant rat model. In addition, a distinction will be made between the role of plasma carnosine vs muscle carnosine in preventing early-stage metabolic stress.

5.3. Carnosinase and diabetes

Serum carnosinase has first been linked to diabetes by Janssen and coworkers in relation to diabetic nephropathy (DN) (Janssen 2005). DN is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli, which is on his turn mainly provoked by high blood glucose levels. It is one of the most severe microvascular complications of type 1 and type 2 diabetes and has become the leading cause of end-stage renal failure in the western world (Ritz 1999).

There is a large body of evidence that DN has a genetic component (Seaquist 1989; Fava 2002) and in 2002 the group of Janssen et al. demonstrated that a region on chromosome 18q22.3-q23 was associated with the development of DN (Vardarli 2002), which is later confirmed by others (Bowden 2004). In a next study (Janssen 2005), they refined their search by looking into the genes related to the pathophysiology of microvascular disease and focused on the inhibition of oxidative stress and AGEs. In this light, they discovered the interesting combination of 1) carnosine having anti-oxidative and anti-glycating properties (cfr supra) and 2) the carnosinase genes CNDP1 and CNDP2 (encoding the carnosine-degrading enzymes CN1 and CN2) being located in this region. As consequence, the link between polymorphisms in the carnosinase genes (CNDP1 and CNDP2) and the development of DN was investigated in 5.500 type 1 and type 2 diabetic patients (European Caucasians and Arabs) and a significant association was found with marker D18S880, a trinucleotide repeat (CTG) in CNDP1 exon 2, encoding the number of leucine repeats (the 5, 6, 7 or 8 leucine allele) (figure 11).

Figure 11. Schematic representation of the different alleles observed in the leucine-repeat region of the serum-carnosinase gene. Shown are the nucleotide and amino acid sequences (Zschocke 2006).

Diabetic patients with two copies of the 5 leucine allele were less susceptible to DN. This association was then confirmed by an independent study in 552 diabetic type 2 European Americans (Freedman 2007), however other studies did not find an association in type 1 diabetic patients (Wanic 2008) or found that the association in type 2 diabetic patients is sex-specific (only present in women) (Mooyaart 2010). To strengthen their hypothesis, Janssen and colleagues showed that the homozygosity for the 5-leucine allele was associated with the lowest CN1 activity and vice versa, the more CTG repeats in CNDP1 (the 6, 7 or 8 leucine allele), the higher the CN1 activity (Janssen 2005). Noteworthy, this was only demonstrated in healthy individuals.

These genetic data in human patients introduced **the working mechanism** why the 5-5 CNDP1 genotype decreases the risk in developing DN: the 5-5 CNDP1 genotype is related to the lowest carnosinase activity (Janssen 2005; Mooyaart 2010; Everaert 2012), which is thought to provide high(er) levels of plasma carnosine (Everaert 2012), and thus protecting tissues (such as kidneys) from ROS, RCS, AGEs, ALEs, …(Aldini 2005; Boldyrev 2006), whereby the risk of diabetes-related complications is decreased (figure 12).

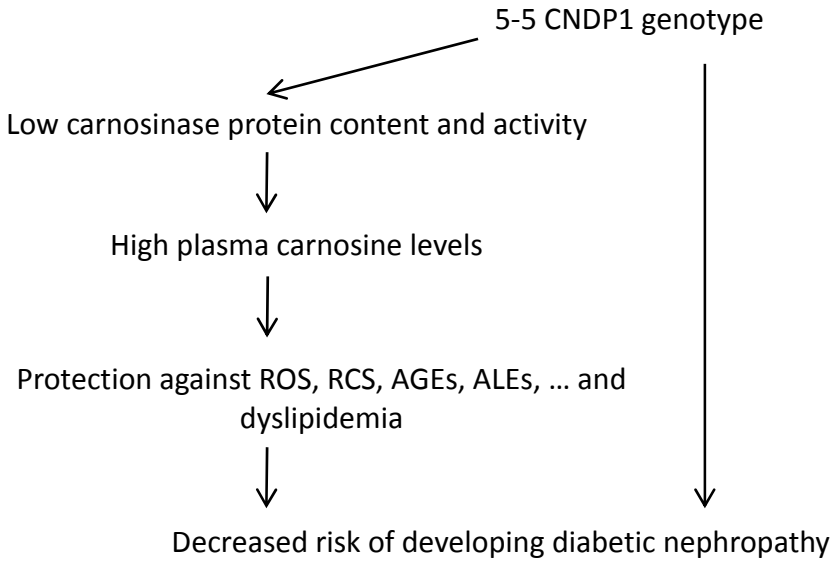

Figure 12. Proposed physiological mechanism for the protective effect of the 5-5 genotype on the development of diabetic nephropathy. Abbreviations: ROS: reactive oxygen species, RCS: reactive carbonyl species, AGEs: advanced glycation end-products, ALEs: advanced lipoxidation end-products, CNDP1: serum carnosinase gene (based on Janssen, 2005).

In this light, Sauerhofer et al. (2007) investigated this hypothesis by manipulating serum carnosine levels of db/db mice. First, endogenous L-carnosine serum levels were artificially lowered by overexpressing the human CN1 enzyme (transgenic mice), second, diabetic mice were given L-carnosine in their drinking water. As hypothesized, fasting plasma glucose as well as HbA1c levels rose significantly earlier and remained higher in transgenic animals. In the opposite approach (carnosine supplementation in db/db mice), diabetes manifested later and milder.

Conclusion: The carnosinase system appears to be important in diabetic patients. However, parts of the *working mechanism* are only confirmed in healthy subjects or in animal models. Therefore, this work will look into some pieces of the carnosinase-diabetes puzzle, by investigating whether low carnosinase activity/content is protective for diabetes related complications (with the focus on a disturbed lipid profile) in diabetic type 2 patients. In addition, the behavior of CN1 in diabetic type 2 patients will be explored (the determinants of CN1 will be discussed for a healthy population in chapter 6.2. of the introduction).

6. DETERMINANTS OF CARNOSINE-CARNOSINASE SYSTEM

Before investigating whether or not the carnosine-carnosinase system is altered in a diabetic/obese population, it is necessary to gain insight into the already known or putative determinants of carnosine content and loading in skeletal muscle and carnosinase concentrations in circulation in a healthy population. The determinants will be discussed below.

6.1. Determinants muscle carnosine content & loading

6.1.1. Determinants muscle carnosine content

Research about the determinants for baseline muscle carnosine content or about the manipulation of muscle carnosine content is the past two decades strongly driven by the sports field. Indeed, high levels of muscle carnosine favor certain types of exercise performance (cfr chapter 4.6.2.). Recently, studies on muscle carnosine content in clinical settings are rising, because the biochemical properties of carnosine suggest a therapeutical potential (cfr supra).

There is a large variation in muscle carnosine content between humans, i.e. the lowest reported value (~10 mmol/kg dw) is about 4 times lower than the highest reported value (~40 mmol/kg dw) at baseline (Parkhouse 1985; Harris 1998; Harris 2006). However, the intra-individual variation in muscle carnosine is rather small (Baguet 2009) and high correlations are found within monozygotic twin pairs (Baguet 2012). Below, the determinants that are responsible for the large inter-individual variability will be described.

Muscle fiber type. Already in 1985, Parkhouse and colleagues found that sprint athletes had remarkably higher muscle carnosine levels compared to untrained subjects and marathoners, in addition, a low but significant correlation was found between carnosine levels and fast-twitch fiber composition (Parkhouse 1985). In 1998, Harris and co-workers analyzed carnosine content in single muscle fibers (determined by ATPase technique) from biopsies of the m. vastus lateralis with high-performance liquid chromatography and found that the mean carnosine concentration was twice as high in type II (IIa and IIx) compared with type I muscle fibers (figure 13), which was later confirmed by others (Hill

2007; Tallon 2007; Kendrick 2009). Finally, this 'fiber type specificity' can be extrapolated to the level of 'muscle type', as muscles with a higher proportion of fast glycolytic type II fibers (m. gastrocnemius) possessed higher levels of carnosine compared to muscles possessing predominantly slow oxidative type I fibers (m. soleus) (Derave 2007). Noteworthy, the results were obtained by proton spectroscopy (^{1}H-MRS), which is the first time that muscle carnosine content could be quantified non-invasively, ultimately leading to a very reliable method to estimate muscle fiber type composition (Baguet 2011a).

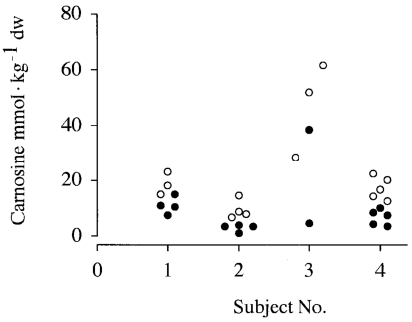

Figure 13. Carnosine content of type I (•) and type II (○) muscle fibres of the vastus lateralis of four subjects (Harris 1998).

Age and sex. Muscle carnosine content increases until the age of ~16 years (adolescence), where after it decreases again until the age of ~70 years (figure 14), unfortunately there are no data available before the age of 9. At all ages, muscle carnosine content is 22-82% higher in men compared to women (Mannion 1992)(Everaert 2011), except for the prepubertal phase where this sexual dimorphism was not yet present (Baguet 2012) (figure 14).

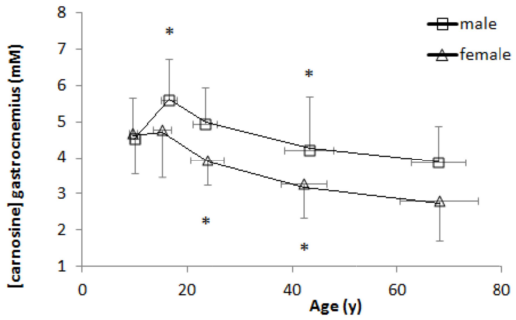

Figure 14. Muscle carnosine concentration (mM) in gastrocnemius muscle in prepubertal children (n=45), adolescents (n=64), young adults (n=91), middle adults (n=39) and eldery (n=24). Data are means ± SD. * Different from the previous age group ($p<0.05$) (Baguet 2012)

Muscle fiber type composition can partly provide an explanation for this age-sex difference in muscle carnosine content, because women and elderly are characterized by a smaller cross-sectional area (CSA) of predominantly fast-twitch fibers (Simoneau 1989). In addition, there are no sex differences in fiber type distribution between boys and girls until the age of 8 (Vogler 1985) and this is the only age group with no difference in muscle carnosine content. Moreover, Tallon et al. demonstrated that carnosine content is equal in muscle fibers of the same kind between men and women (Tallon 2007).

However, there are some indications that *androgens* take part as well in explaining the age-sex differences in muscle carnosine content, irrespectively of muscle fiber type. Penafiel et al. (2004) demonstrated that castrated mice have 40% less muscle carnosine compared to the uncastrated animals and testosterone administration in female mice increased muscle carnosine content by 268% (Penafiel 2004). This increase can never be explained by changing CSA of fiber types as it has been shown that testosterone supplementation has limited or no impact on CSA of fiber types (Sinha-Hikim 2006).

Logically, as beta-alanine is present in meat and fish (under the form of anserine and carnosine), it can be suggested that *dietary habits* (being a vegetarian or eating little/much meat) influence muscle carnosine content (the average daily intake of beta-alanine from an omnivore western diet equals approximately ~330 mg/day (Everaert 2011). However, data are still controversial. Everaert et al. (2011) has demonstrated that 12 male long-term (> 8 years) vegetarians had lower muscle carnosine levels compared to male omnivores (-20%), whereas switching from an omnivorous diet to a vegetarian diet for 5 weeks did not alter muscle carnosine content (Baguet 2011a). Probably the intervention period was too short to induce a significant decrease, however, the impact of switching to a vegetarian diet is still unclear.

Conclusion: The main determinants of muscle carnosine content are muscle fiber type, sex and age. However, it has to be determined whether diabetes can alter muscle carnosine content as well (as suggested from the quenching theory above, cfr. 5.2.). Therefore, this work will investigate skeletal muscles of different obese/diabetic rodent models.

6.1.2. **Determinants muscle carnosine loading**

After it has been discovered that high muscle carnosine levels enhances high–intensity exercise performance (chapter 4.6.2.), follow-up research has been focusing on increasing or 'loading' muscle carnosine. As said, the clinical field is watching because it has been suggested that muscle carnosine is 'possibly' decreased in a diabetic state.

The far most efficient way to load muscle carnosine is by beta-alanine supplementation (Harris 2006). As described above, beta-alanine is the rate-limiting factor because L-histidine is present in sufficient amounts in the muscle, while beta-alanine is present in very low concentrations. An efficient beta-alanine supplementation protocol to augment muscle carnosine content was originally established by Harris and colleagues (Harris 2006). The latter and subsequent studies have shown that chronic oral supplementation of 1.6 to 6.4 g/day of beta-alanine will generally lead to increases in muscle carnosine content of ~15-85% in 4 to 12 weeks (Hill 2007; Baguet 2009; Del Favero S 2012; Stellingwerff 2012). In this line, Stellingwerff et al. (2012) demonstrated that the total amount of beta-alanine consumed (rather than the daily dose) predicts both the relative and absolute increase in muscle carnosine, with an increase of 2.01 mmol/kg ww per 100g of consumed beta-alanine. Noteworthy, there is a large *variability* in the response of beta-alanine supplementation, which has been demonstrated by Baguet et al. (2009). Here, untrained males received 4.8g beta-alanine per day for 5-6 weeks, high responders showed a 55% increase in muscle carnosine, whereas low responders only 15%.

Although a lot of research has been performed, there are still some crucial elements missing. First, limited information exists about factors being responsible for the high variability in muscle carnosine loading. It can be questioned whether the factors influencing baseline carnosine content (sex, muscle fiber type, diet, androgens) would also influence muscle carnosine loading. In addition, little is known about the effect of body weight, fat-free mass and baseline carnosine content during loading. Second, up until now, no ceiling level has been reported. Finally, little is known about the metabolic fate of chronically ingested beta-alanine. When calculating the percentage of the total ingested beta-alanine dose (over a prolonged period) that is actually incorporated into

muscle carnosine, this is less than 6%. However, there is no information available about long-term urinary beta-alanine excretion.

When relating muscle carnosine loading to diabetes, we can question whether insulin (a key hormone in diabetes) can stimulate beta-alanine uptake in muscle, by triggering the sodium dependent beta-alanine transporter Taut. This would be similar to the metabolism of creatine, another popular nutritional supplement that enhances performance through muscle loading. Here, insulin stimulates the sodium-dependent creatine transporter as well (Green 1996; Steenge 1998; Sweeney 1998)(mechanism will be schematically represented in part III, figure 2).

Upon discontinuation of beta-alanine supplementation, muscle carnosine levels will gradually return to baseline, pre-supplementation levels in 6-20 weeks (a long wash-out period) (Baguet 2009; Stellingwerff 2012). A first attempt to establish a maintenance dose (to keep up elevated muscle carnosine levels) was published by Stellingwerff et al. (2012). They demonstrated that after an initial loading protocol of 3.2g beta-alanine/day for 4 weeks, a further ~30% increase in muscle carnosine occurred after 4 weeks of taking 1.6g beta-alanine per day. We can conclude that the optimal maintenance dose must be found between 0 and 1.6g beta-alanine per day. Finding a maintenance dose is particularly important for long-term studies about the effects of chronically elevated muscle carnosine levels on sports performance *or* in clinical situations. Therefore, this work will investigate 3 intermediate doses: 0.4g/day, 0.8g/day and 1.2g/day.

Conclusion: Muscle carnosine loading is accomplished by beta-alanine supplementation, however 1) a large variability exists in muscle carnosine loading response and 2) it is unknown how much beta-alanine is retained by the body during chronic supplementation. Therefore, this work will 1) focus on possible determinants such as the effect of insulin, sex, body weight and fat-free mass in the loading phase and 2) urinary excretion and muscle accumulation of beta-alanine will be explored during chronic supplementation. Finally, this work will propose a maintenance dose to keep up elevated muscle carnosine stores.

6.2. Determinants serum carnosinase

Studies investigating the enzyme serum carnosinase are mainly coming from the clinical field, as the relevance of CN1, or its gene (CNDP1), is predominantly related to diabetes or diabetes-related complications (cfr supra).

Similar to muscle carnosine content, serum carnosinase describes a considerable variation between individuals (Lenney 1982). The stability within individuals throughout the day is remarkably stable (Peters 2011b), however, the stability over weeks/months is not known. Below, the known or putative determinants are described. Notably, these are only applicable for humans, as CN1 is not present in serum of rodents.

Age and sex. CN1 activity is found in small amounts in newborns (Bando 1984) and raises gradually with age until adolescence is reached, with no further change throughout life (Lenney 1982), however, CN1 protein content does not differ between children and adults, pointing towards different CN1 conformations in the respective age groups (Peters 2010). Once adolescence is reached, a sexual dimorphism appears, i.e. women have higher levels of both CN1 activity and content compared to men (Bando 1984; Peters 2010; Everaert 2011).

CTG repeats. This is discussed above (subchapter 5.3.). However, briefly, Janssen et al. (2005) reported that a particular genotype is associated to CN1 activity. The five leucine allele was associated with the lowest CN1 activity levels (Janssen 2005). Although this association appears to be relevant for diabetic patients (as explained above), it is not (yet) investigated in this specific population, which represents a big gap in the literature.

N-glycosylation is recently reported as another determinant and is directly related to diabetes. N-glycosylation is a subclass of glycosylation (the most frequent posttranslational modifications of macromolecules) and contains the attachment of a sugar molecule ($glucose_3$-$mannose_9$-N-$acetylglucosamine_2$, which is called an N-glycan) to a nitrogen atom in an amino acid residue of a protein. This type of linkage is important for both the structure and function/activity of proteins and takes place in the endoplasmic reticulum. CN1 is susceptible for enzymatic N-glycosylation, as it has three putative N-glycosylation sites (Teufel 2003). Knowing this, Riedl et al. (2010) stepwise deleted the 3

putative N-glycosylation sites in transfected cells and in these cells CN1 secretion and activity was gradual inhibited. Next, Riedl et al. (2010) suggested that hyperglycemia would increase CN1 secretion/activity because of increased N-glycosylation. *Hyperglycemia* triggers the hexosamine pathway, leading to elevated UDP-N-acetylglucosamine (GlcNAc) concentrations (figure 8) (Brownlee 2001). As Sasai et al. has demonstrated that the level of UDP-GlcNAc is a critical factor in the production of N-glycans, which on his turn increases N-glycosylation (Sasai 2002), these increased levels of UDP-GlcNAc will probably enhance CN1 secretion/activity. Indeed, they demonstrated that 11 homozygous diabetic (type 1 and type 2) patients for the 5-leucine allele had increased CN1 activity levels (~20%) compared to 15 homozygous healthy controls (Riedl 2010). This may be a disadvantage for diabetic patients, as it is suggested that high CN1 activity levels counteract the protective circulating dipeptide carnosine (upon dietary ingestion).

Other determinants. CN1 belongs to the family of the M20- metalloproteinases. As the name speaks for itself, these proteinases use metal ions for its catalytical activity (Lenney 1985; Teufel 2003). In addition, there is a competitive inhibition between the substrates of carnosinase, being not only carnosine, but also homocarnosine and anserine. However, it is uncertain whether this is relevant in vivo (Peters 2010; Peters 2011b).

Combining the two latter chapters about muscle carnosine content and serum carnosine activity, it seems that muscle carnosine content and serum carnosinase activity are related. For example, women and elderly have low muscle carnosine levels, but display a high activity of CN1. However, Everaert et al. (2011) has shown that there is no inverse relationship between carnosine content in muscle and carnosinase activity in circulation within subjects. Similarly, the polymorphism of the CNDP1 gene does not predict muscle carnosine levels. The authors explain this finding to a different compartimentation because carnosine is mainly present in muscle, whereas carnosinase (CN1) is present in circulation.

Conclusion: Age, sex and CTG repeats are the dominant determinants of serum carnosinase, although demonstrated in a healthy population. This work will examine whether the determinants age and sex still hold for a diabetic population and whether prolonged hyperglycemia (HbA1c) is positively correlated with serum carnosinase in a large cohort diabetes type 2 patients. In addition, the effect of an exercise program (aiming to attenuate hyperglycemia) on serum carnosine content and activity will be examined.

7. EXPERIMENTAL AIMS AND OUTLINE OF THE THESIS

Carnosine is a dipeptide with several biochemical and physiological properties presumably relevant in a diabetic state. The introduction has put forward some arguments for **an altered carnosine-carnosinase system** in a glucose intolerant state. In addition, evidence has been given **that carnosine administration** counteracts the development of diabetes or diabetes-related diseases.

In part II, animal and human studies provided an answer whether or not the carnosine-carnosinase system is truly affected in a diabetic state. In addition, it was demonstrated how carnosine (or beta-alanine) administration can be protective in an early stage of metabolic disease. Therefore, five studies were performed (figure 15):

First, it was investigated how muscle carnosine loading by beta-alanine supplementation takes place under normal physiological conditions and whether insulin (one of the key hormones in diabetes) plays a role. This was indirectly investigated by comparing long-term beta-alanine supplementation in between the meals (out-phase) or during the meals (in phase) (**study 1**).

In line with this, a theoretical explanation was given for net carnosine turnover in different situations (baseline, loading, washout and maintenance) and the beta-alanine dose was proposed in order to maintain moderately elevated muscle carnosine levels (relevant for athletes and presumably relevant in clinical populations) (**study 2**).

In a next study (**study 3**), the effect of carnosine supplementation in a high-fat rat model was investigated to elucidate the early metabolic effects of carnosine administration. In addition, a critical unanswered question was the site of action of carnosine, being either carnosine levels in tissues (with focus on muscle) or in plasma. Therefore, this was investigated using oral carnosine versus beta-alanine supplementation, since beta-alanine can increase muscle carnosine levels, without a concomitant increase in plasma carnosine.

The **fourth study** originated from the uncertainty that muscle carnosine levels are actually decreased in a diabetic state, since we did not observe decreased muscle carnosine levels in our mild high-fat diet rat model (study 3). Therefore, rodents receiving various degrees of high-fat diets, resulting in various degrees of glucose intolerance, were examined for

their muscle carnosine content. Ultimately providing an answer whether muscle carnosine is actually decreased in a glucose intolerant state.

Finally, CN1 content and activity was investigated in 243 diabetic type 2 patients, before and after a 6-month intervention, being either an exercise program (aerobic, resistance or combined exercise) or remaining sedentary (**study 5**). This design allowed us to explore the behavior of baseline CN1 in a diabetic state and whether or not it could be influenced by exercise training. In addition, the hypothesis 'low carnosinase is protective in the development of diabetes related metabolic disturbances' was tested.

In part III, the main findings of the thesis were summarized and discussed to finally end with a brief conclusion.

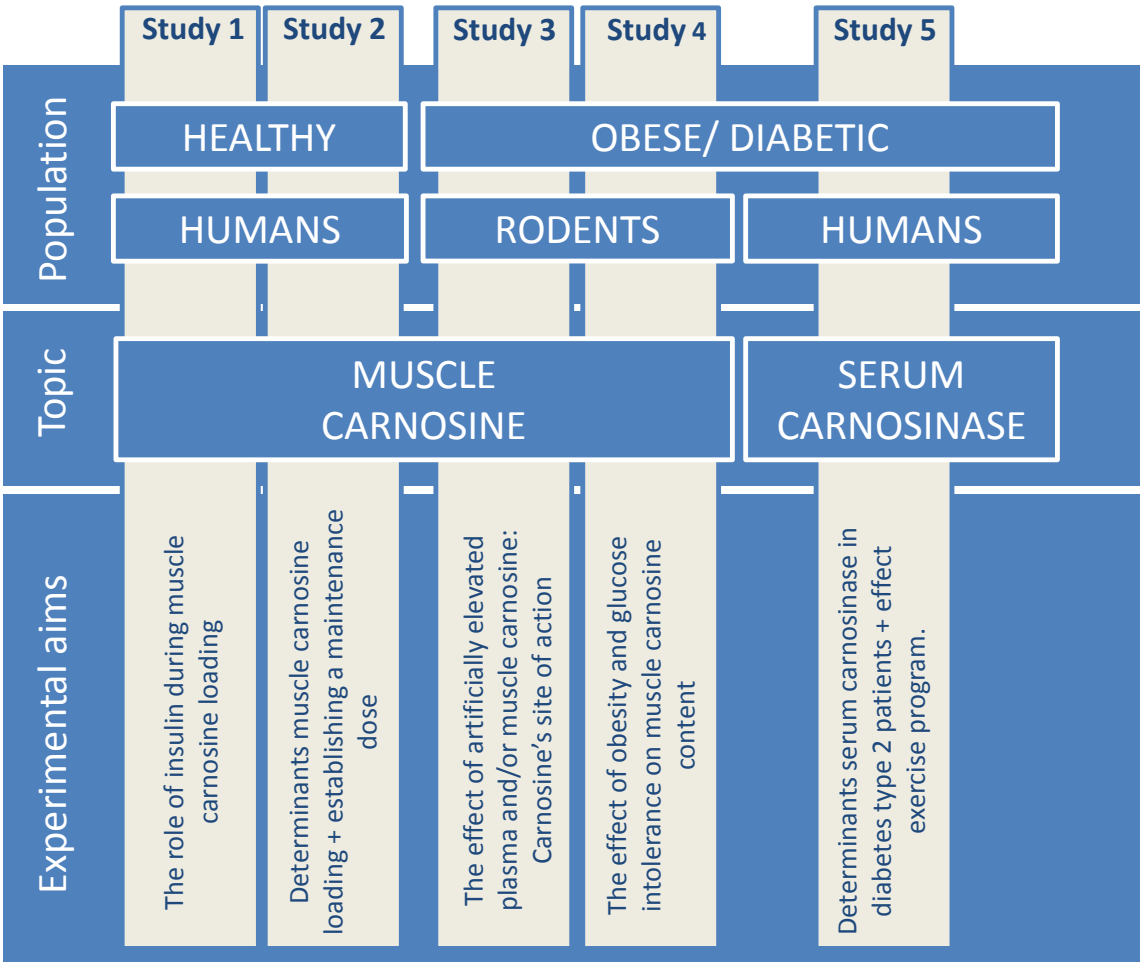

Figure 15: Schematic representation of the experimental aims and outline of the thesis.

PART II

Original Research

Study 1

MEAL AND BETA-ALANINE CO-INGESTION ENHANCES MUSCLE CARNOSINE LOADING

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ABSTRACT

Introduction. Beta-alanine (BA) is a popular ergogenic supplement as it can induce muscle carnosine loading. We hypothesize that, by analogy with creatine supplementation, 1) an inverse relationship between urinary excretion and muscle loading is present, and 2) the latter is stimulated by carbohydrate-and protein-induced insulin action.

Methods. In study A, the effect of 5 weeks slow-release BA (SRBA) supplementation (4.8g/day) on whole body BA retention was determined in 7 men. We further determined whether co-ingestion of carbohydrates and proteins with SRBA would improve retention. In study B (34 subjects), we explored the effect of meal-timing on muscle carnosine loading (3.2g/day during 6-7 weeks). One group received pure BA (PBA) in between the meals, the other received PBA at start of the meals, in order to explore the effect of meal-induced insulin release. Further, we compared with a third group receiving SRBA at start of the meals.

Results and conclusion. Orally ingested SRBA has a very high whole body retention (97-98%), that is not declining throughout the 5 weeks supplementation period, nor is it influenced by co-ingestion of macronutrients. Thus, a very small portion (1-2%) is lost through urinary excretion, and equally only a small portion is incorporated into muscle carnosine (~3%), indicating that the majority of ingested BA is metabolized (possibly through oxidation). Secondly, in soleus muscles, the efficiency of carnosine loading is significantly higher when PBA is co-ingested with a meal (+64%), compared to in between the meals (+41%), suggesting that insulin stimulates muscle carnosine loading. Finally, chronic supplementation of SRBA versus PBA seem equally effective.

Keywords: beta-alanine retention, ergogenic supplements, carnosine synthase

INTRODUCTION

Human skeletal muscles contain carnosine (β-alanyl-L-histidine), a cytoplasmic dipeptide, in relatively high concentrations (~ 5 mmol/kg wet weight) (Harris 1990). Chronic supplementation of beta-alanine (BA), the rate-limiting precursor for carnosine synthesis, can significantly increase muscle carnosine content by 40 – 80% when ingested in doses of ~ 1.6 – 6.4 g/day for ≥ 4 weeks (Harris 2006; Derave 2007; Hill 2007b; Baguet 2009; Stellingwerff 2012). BA supplementation has recently become an intensive research topic, not only in the exercise and performance-enhancement field, but also in clinical populations with chronic glycoxidative and inflammatory damage. An increase in muscle carnosine is related to an increase in performance for high-intensity exercise (Hill 2007a; Baguet 2010; del Favero 2012). Moreover, low muscle carnosine levels are found in certain clinical populations such as diabetes type 2 and elderly (del Favero 2012; Gualano 2012), which may suggest that carnosine loading could be beneficial. These findings are assisted by in vitro and preclinical research suggesting a multitude of physiological roles for carnosine, such as a regulator of muscle excitation-contraction coupling via increasing calcium sensitivity, proton buffer, antioxidant, an inhibitor of protein glycation, as extensively reviewed by Boldyrev (2012) and Derave et al. (2010).

In order to understand the mechanism of muscle carnosine loading, it is necessary to gain insight into the metabolic fate of chronically ingested BA in humans. Decombaz et al. (2012) acutely supplemented pure (PBA) and slow-release (SRBA) BA and showed a very high whole body retention for both forms, respectively 96.3% and 98.9%. SRBA was developed in order to avoid high peak concentrations of BA in the blood, which is associated with side-effects such as paresthesia, however its efficiency for muscle carnosine loading has never been directly compared with PBA. When calculating the percentage of the total ingested BA dose (PBA or SRBA ingested over a prolonged period) that is actually incorporated into muscle carnosine, this is less than 6% (Hill 2007b; Baguet 2009; Stellingwerff 2012). Therefore, we expect that the retention of BA, albeit nearly maximal upon a first oral challenge (Decombaz 2012), will decrease during chronic supplementation. This would be similar to two other popular nutritional supplements that enhance performance through muscle loading, namely creatine and carnitine, which are similar small nitrogen-containing molecules with high natural abundance in skeletal

muscle. Even though the biochemical mode-of-action with respect to involvement in energetic pathways leading to exercise performance enhancement is different between creatine, carnitine and carnosine, the mechanism of accumulation in muscle is strikingly similar: the muscular loading is mainly limited by the transsarcolemmal transport system and the transport is dependent on and driven by sodium ion co-transport. The retention of creatine and carnitine is high in a first ingestion, but subsequently decreases during chronic supplementation. After 3 days (20 g/day) creatine retention decreases from 53% to 41% (Green 1996a) and after 14 days (3 g/day) carnitine retention decreases from 98% to 90% (Stephens 2007).

Interestingly, the urinary loss of creatine and carnitine seems to be inversely related to the muscle loading efficiency, or in other words, when the muscle pools get saturated and reach maximal levels, the compounds (creatine, carnitine) start to appear more abundantly in the urine (Green 1996b; Stephens 2007). According to this rationale, the search for new interventions to improve the muscle loading efficiency can start with looking at interventions that decrease urinary excretion, as a proxy of muscle accumulation. Indeed, when large amounts of carbohydrates are co-ingested with creatine or carnitine, its urinary excretion decreases (Green 1996a; Stephens 2007). The follow-up research then demonstrated that more creatine and carnitine is accumulated in the skeletal muscle under the influence of carbohydrate- and protein-mediated insulin release (Green 1996b; Steenge 1998; Wall 2011). Insulin stimulates the sodium-dependent creatine (CreaT) and carnitine (OCTN2) transporter, secondary to its action of increasing sarcolemmal Na+/K+-ATPase pump activity and therefore transmembrane Na+ flux (Sweeney 1998; Clausen 2003). Because it has been shown that the most important BA transporter (TauT) is strongly dependent on sodium and chloride co-transport (Bakardjiev 1994), it is equally expected that muscular BA uptake, and hence carnosine loading, is enhanced by insulin action.

Therefore, this study was designed to investigate the distribution of chronically ingested BA, by looking at urinary BA excretion and muscle carnosine loading. Secondly, does co-ingestion of carbohydrates and proteins has the potential to alter this distribution of BA in the body? And finally, is SRBA more efficient concerning muscle carnosine loading compared to PBA?

MATERIALS AND METHODS

Subjects

Forty-five healthy non-vegetarian men and women participated in the present studies (study A and B), which were approved by the local ethical committee (Ghent University Hospital, Ghent, Belgium). All gave their written informed consent to take part in the studies and were aware that they were free to withdraw from the experiment at any point.

Study protocol

Study A (represented in figure 1). Seven men (age: 22.1 ± 1.3 yr, body weight: 80.7 ± 11.8 kg) were supplemented for 5 weeks with 4.8g slow release beta-alanine (SRBA) (3x/day 2 tablets of 800 mg, PowerBar). Retention was measured weekly (by ingestion of 1.6g SRBA in fasted state and collection of urine up to 6h post-ingestion) and calculated by the amount of BA not excreted in urine. Total urinary volume was determined and urine aliquots were collected in EDTA-coated tubes and stored at -20°C for further HPLC analysis of BA. During the 6h period, the subjects were withdrawn from food, but had at liberty access to water. From week 2, subjects performed the retention twice on consecutive days: in the first condition subjects took 1.6 g SRBA in fasted state (= FASTED), in the second condition subjects took 1.6g SRBA together with the intake of 2 energy carbohydrate-rich bars of PowerBar (=CHO) (PowerBar Europe GmbH). To sustain elevated levels of plasma insulin, the first carbohydrate-rich bar (Energize C2 max: 39.1g CHO + 5.8g protein) was taken 10min after BA intake and the second protein rich bar (Protein Plus: 22.2g CHO + 16.6g protein) 55min after BA intake. According to Steenge et al. (2000) , we can assume that the ingestion of 61.3 g CHO + 22.4 g protein would elevate plasma insulin above 70 mU/L.

Study B (represented in figure 1). In order to determine the effect of meal-induced insulin release on muscle carnosine loading, we first aimed to design a protocol in which the area under the curve (AUC) for plasma insulin and BA would either overlap with each other or would be completely separated from each other. For this purpose we subjected four participants to a small acute pretest (age: 22.5 yr, body weight: 76.6 kg, 2 males and 2 females).

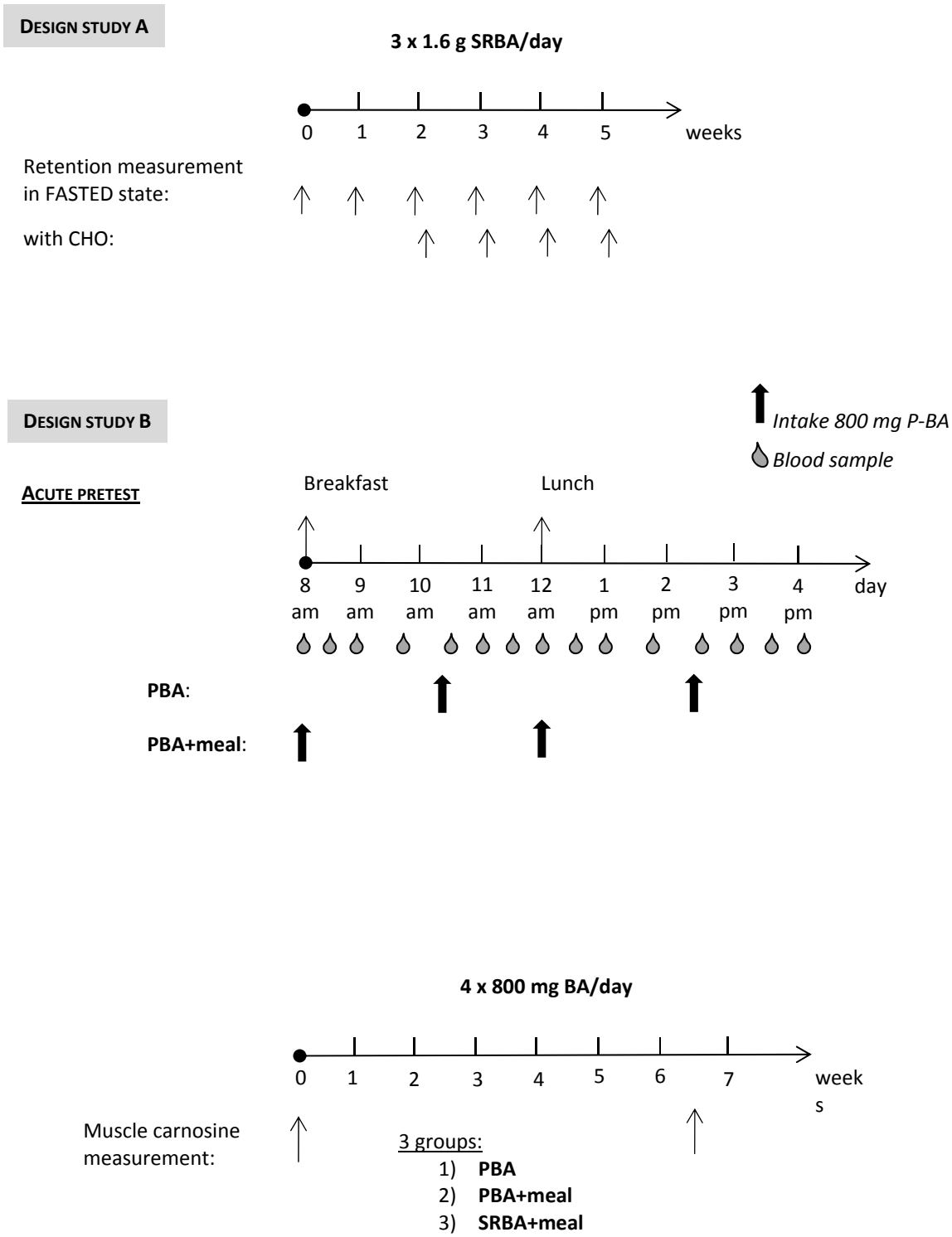


Figure 1: Schematic representation of study design A and B

Over a period of 8 hours, subjects ingested 2 meals, starting with a breakfast at 8 am (850 kcal: 74% CHO, 14% P, 12% F) and a lunch at 12 pm (950 kcal: 66% CHO, 13% P, 21% F). Both meals were standardized and contained no meat. Breakfast consisted of 4 slices of white bread with chocolate-hazelnut paste, 200ml semi-skimmed milk, 1 banana and 125gr fruit yogurt. Lunch contained a 150gr baguette topped 40gr young cheese, 30gr mayonaise and vegetables with a 33cl orange juice. Subjects all drank 1,5L water during the 8 hours. Two subjects (1 male, 1 female) ingested PBA (2 x 800 mg) interspersed in between meals (at 10.30 am and 2.30 pm: *'PBA'*) and two other subjects ingested PBA at the start of the meals (8 and 12 am: *'PBA+meal'*). Every 30 to 45 minutes venous blood samples (EDTA coated tubes and serum tubes for respectively BA and insulin determination) were taken from a catheter, placed in an antecubital vein. We defined the AUC where elevated plasma BA values (> 30 µM) coexisted with moments of elevated plasma insulin levels (> 25 µU/ml) induced by meals.

We subsequently adopted this protocol of the pretest, i.e. comparison of ingesting PBA either during or interspersed between meals, into a full study lasting 46 days. In addition, a third experiment group was included that chronically ingested SRBA in the same dose. Thirty-four subjects (age: 19.4 ± 1.0 yr, body weight: 66.6 ± 9.9 kg), both males (n = 16) and females (n = 18) were randomized into three sex-balanced groups. All groups received 3.2g BA daily, divided over 4 intakes of 800 mg each, with at least 3h between intakes. The first group, *'PBA',* received PBA between the meals/snacks (CarnoSyn beta-alanine powder in 400 mg gellules, Natural Alternatives International (NAI), San Marcos, USA). Subjects were not allowed to eat 150 minutes before and 90 minutes after each intake (4 daily) of BA, throughout the 46 days. The second group, *'PBA+meal'*, received PBA at the start of each meal (3x/day) and a fourth pill had to be taken with a carbohydrate-rich snack. The last group, *'SRBA+meal'*, took SRBA (CarnoSyn SR tablets, NAI) 30 minutes before a meal or snack. Before and after the supplementation period muscle carnosine content was measured and during supplementation subjects had to fill in a diary for the registration of every BA and meal/snack consumption. Finally, all subjects completed a questionnaire of their meat and fish consumption during the first 2 weeks of the study in order to quantify daily dietary BA ingestion, as described by Baguet et al. (2009).

Determinations in plasma and urine samples

The concentration of BA in plasma and urine was determined by high-performance liquid chromatography (HPLC). One hundred microliters of EDTA-containing plasma and urine was taken and added with 11.1 µl S-sulfosalicylic acid to deproteinize the samples. These deproteinized samples were dried under vacuum (40°C) for 45 minutes. Dried residues were resolved with 40 µl of coupling reagent: methanol-triethylamine-H2O-phenylisothiocyanate (7:1:1:1) and allowed to react for 20 min at room temperature. Samples were dried again and resolved in 100 µl sodium acetate buffer (10 mM, pH 6.7). The same method was applied for the standard solutions of BA (Sigma, dissolved in deionized distilled water). Derivatized samples (20 µl) were chromatographed on a Waters HPLC system with a Spherisorb C18/ODS2 column (4.6 x 150 mm, 5 µm), ODS2 guard column (80Å, 5 µm, 4.6 mm X 10 mm) and UV detector (wavelength: 210 nm). The columns were equilibrated with buffer A (10 mM sodium acetate adjusted to pH 6.8 with 6% acetic acid), buffer B (60% acetonitrile-40% buffer A) and buffer C (100% acetonitrile) at a flow rate of 0.8 ml/min at 25°C. The limit of quantification was 7 µM. Plasma insulin was determined by electrochemiluminescent assay on a Cobas E411 (Roche Diagnostics, Mannheim Germany).

Determination of muscle carnosine content

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 Baguet et al. (2010). The subjects were lying in supine position on their back and the lower leg was fixed in a holder with the angle of the ankle at 20° plantar flexion. All the MRS measurements were performed on a 3-T 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) of 2.000 ms, echo time (TE) of 30 ms, number of excitations is 128, 1.024 data points, spectral bandwidth of 1.200 Hz, and a total acquisition time of 4.24 min. The average voxel size for the soleus and gastrocnemius muscle was respectively 40 mm x 10 mm x 28 mm and 40 mm x 11 mm x 30 mm. The line width of the water signal for the soleus and gastrocnemius muscle was on average respectively 24.6 Hz and 27.6 Hz, following

shimming procedures. The absolute carnosine content (in millimolar; mM) was calculated as described before by Baguet et al. (2010).

Statistics

A general linear model repeated measures ANOVA was used to evaluate a decrease in BA retention over time. To look for differences between PLA and CHO a paired sample t-test was performed at each time point. To examine the absolute and relative difference in muscle carnosine between '*PBA'* and '*PBA+meal'*, a 2x2 general linear model repeated-measures ANOVA was performed, with absolute/relative increase in muscle carnosine as the dependent variable and muscle group and intervention group as the between-subjects factors. A posthoc independent two sample T-test was performed to study the difference for each muscle separately. To investigate the efficiency of *PBA+meal* versus *SRBA+meal*, the same statistics were performed as above. All analyses were done with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p < 0.05$.

RESULTS

Whole body beta-alanine retention (Study A).

Urinary BA excretion was found to be very low and unaltered throughout the study in all subjects. Of the ingested 1600 mg SRBA, only ~26 mg (range 1 - 75 mg) was excreted in the urine. Whole body BA retention was 97.98% during a first oral challenge with SRBA. Long-term supplementation did not affect the retention (week 1 – 5: 97.98 ± 1.45%, 98.45 ± 1.20%, 98.05 ± 0.85%, 98.53 ± 0.66 %, 98.79 ± 0.59%, respectively, $p > 0.05$). Logically, co-ingestion of SRBA with carbohydrates and proteins did not enhance BA retention (week 2: 98.37 ± 0.92%, week 3: 98.71 ± 0.83%, week 4: 98.54 ± 0.98%, week 5: 97.56 ± 1.74%, $p > 0.05$) (figure 2).

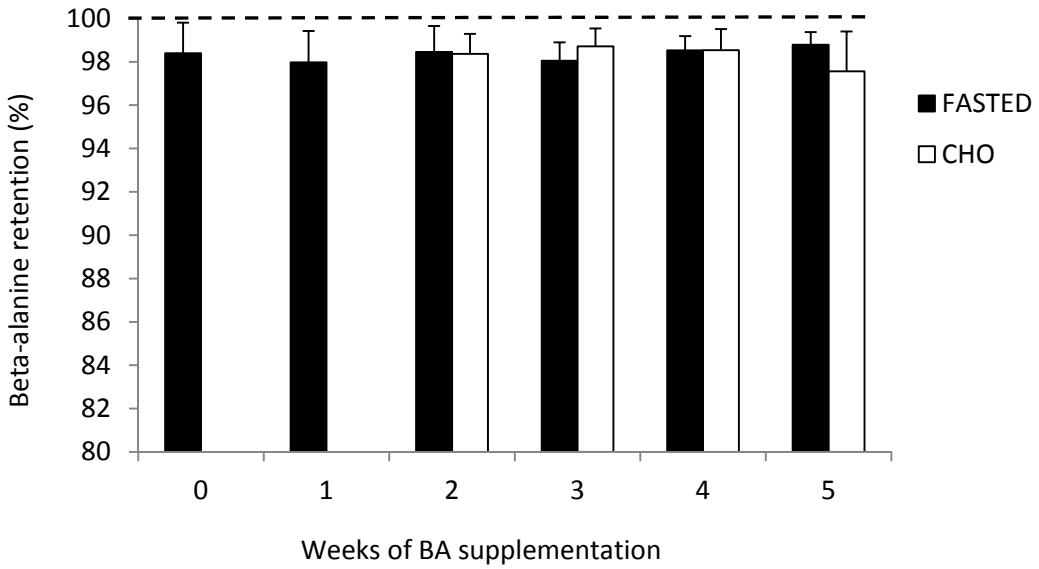


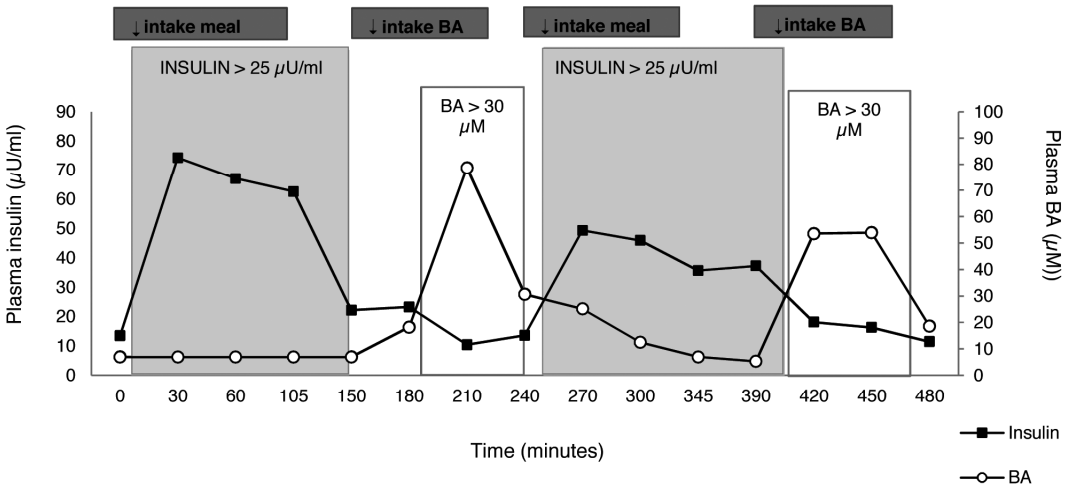
Figure 2: Study A: BA retention during a 5-week supplementation period of 4.8g SRBA/day with (CHO) or without (FASTED) co-ingestion of CHO and proteins. * $p<0.05$ versus CHO.

Plasma insulin and beta-alanine (Study B).

Figure 3 represents the profile of plasma insulin and BA during 8 hours when PBA ingestion is interspersed in between (A) or coincides (B) with meals. In figure 3A, the moments of insulinemia (>25 µU/ml) and beta-alaninemia (> 30 µM) are clearly separated, whereas in 3B the elevations in plasma insulin and BA clearly overlap. This is represented

by respectively 0% and 82% of the total AUC of plasma BA showing elevated BA levels at the moment of hyperinsulinemia.

3A.



3B.

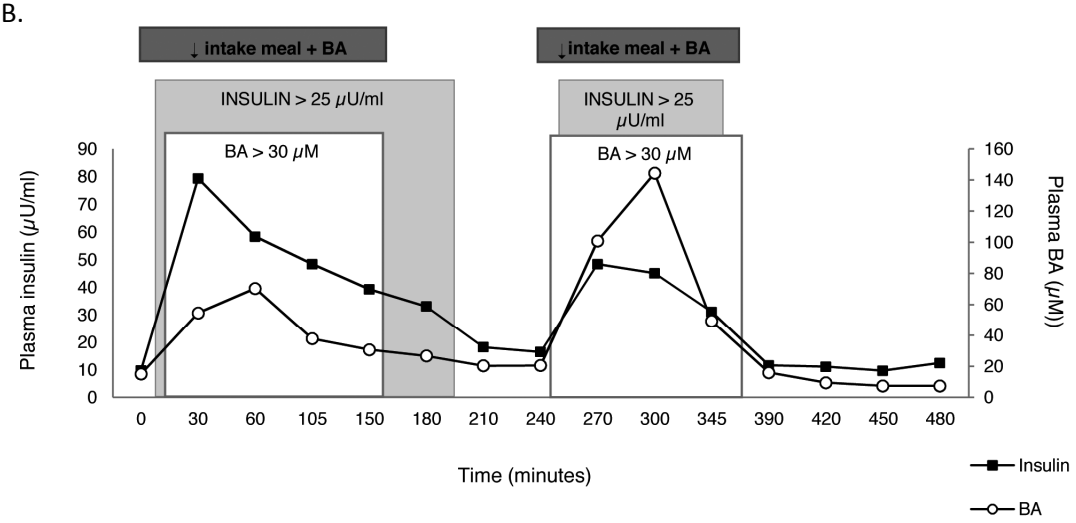


Figure 3: Study B: Blood profile of plasma insulin and beta-alanine (BA) during 8 hours. 3A. average of 2 subjects taking PBA in between the meals (PBA). 3B. average of 2 subjects taking PBA at the start of the meals (PBA+meal).

Muscle carnosine content (Study B).

There were no differences between the groups concerning age, height, weight and total dietary BA intake (table 1) nor concerning baseline carnosine concentration in soleus and gastrocnemius (table 2). Baseline soleus carnosine concentration (3.47 ± 0.87 mM) was lower compared to gastrocnemius muscle (4.51 ± 1.36 mM; $p < 0.001$). In soleus, the absolute (1.98 ± 0.96 mM) and relative (63.6 ± 41.6 %) increase in carnosine was higher

when PBA (4 x 800 mg) was taken at the start of each meal/snack ('PBA+meal'), compared to the group that ingested PBA in between the meals ('PBA', respectively 1.40 ± 0.44 mM and 40.7 ± 14.2 %, table 2 and figure 4) (main effect 'group': p=0,08 ; independent two sample t-test for soleus separately: p=0,049). In gastrocnemius muscle, the effect of meal timing was not present (independent two sample t-test for gastrocnemius separately: p=0.531). The muscle carnosine loading in the group 'SRBA+meal' was not statistically different from 'PBA+meal'.

	PBA	**PBA+meal**	**SRBA+meal**
Number of subjects (M/F)	10 (4/6)	12 (6/6)	12 (6/6)
Age (yr)	19,3 (± 0,9)	19,3 (± 1,1)	19,6 (± 0,9)
Height (cm)	173,0 (± 7,0)	171,7 (± 11,2)	174,3 (± 10,4)
Weight (kg)	68,2 (± 10,1)	66,7 (± 8,8)	65,3 (± 11,3)
Dietary BA intake (mg/day)	213 (± 146)	250 (± 82)	245 (± 93)

Table 1: Baseline values of the three groups of study B. There are no significant differences between the groups. Data are means ± SD.

M. SOLEUS	**[Carnosine] Pre (mM)**	**[Carnosine] Post (mM)**	**Delta (mM)**
PBA	3.54 (± 0.86)	4.94 (± 1.13)*	1.40 (± 0.44)
PBA+meal	3.46 (± 0.93)	5.44 (± 1.02)*	1.98 (± 0.96) †
SRBA+meal	3.43 (± 0.89)	5.14 (± 1.06)*	1.71 (± 0.62)
M. GASTROCNEMIUS	**[Carnosine] Pre (mM)**	**[Carnosine] Post (mM)**	**Delta (mM)**
PBA	4.59 (± 1.66)	6.09 (± 1.73)*	1.50 (± 0.83)
PBA+meal	4.26 (± 0.93)	5.98 (± 0.91)*	1.72 (± 0.61)
SRBA+meal	4.70 (± 1.53)	6.48 (± 1.67)*	1.78 (± 0.99)

Table 2: Muscle carnosine concentrations before and after a 46-days loading protocol (study B) in the three intervention groups. Data are means ± SD. * p<0.05 versus pre. † p< 0.05 versus PBA.

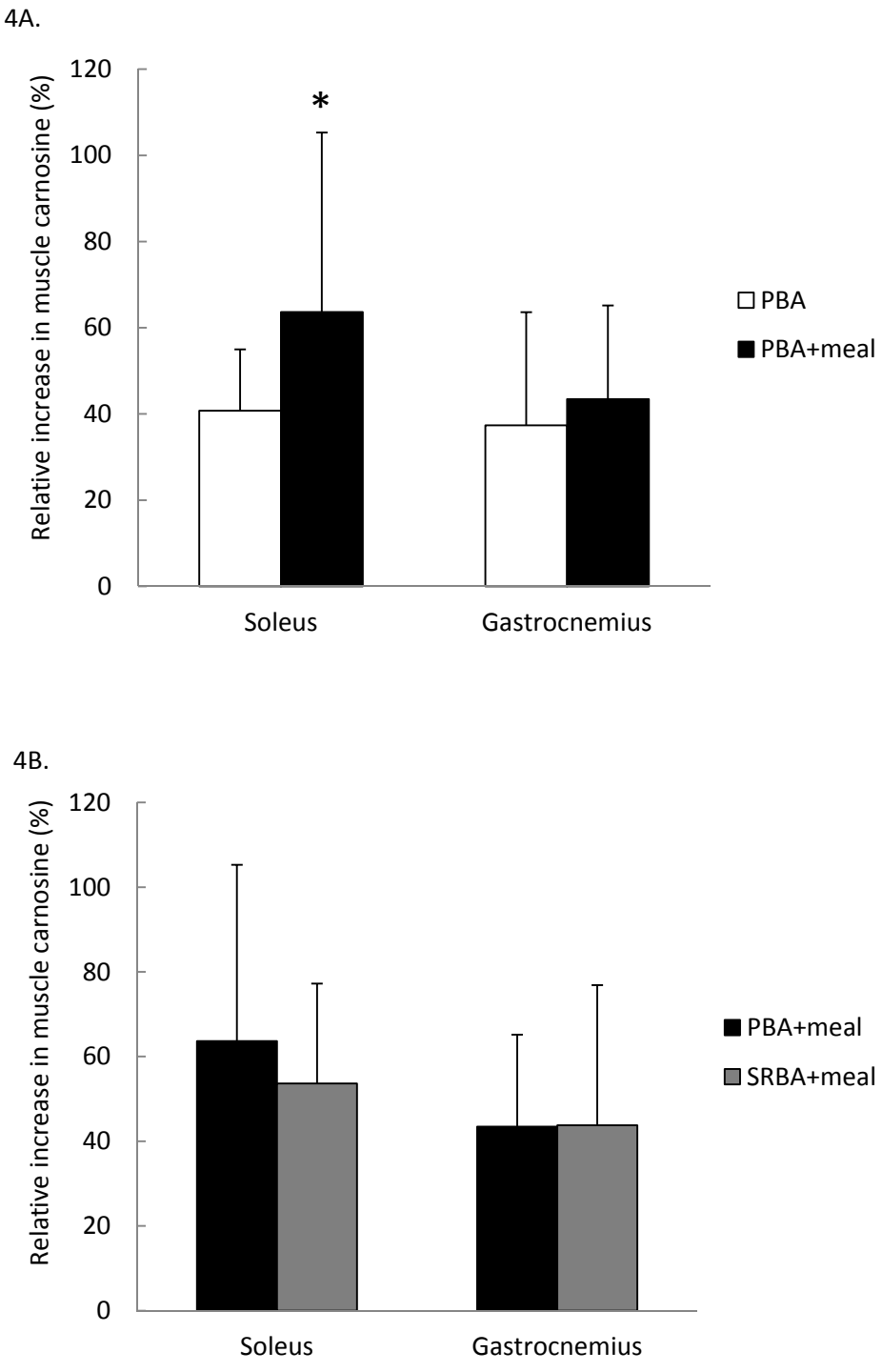


Figure 4: Relative increase in muscle carnosine concentrations before and after a 46-days loading protocol (study B) between the groups 'PBA' and 'PBA+meal' (figure 4A) and 'PBA+meal' and 'SRBA+meal' (fugure 4B). * $p < 0.05$: vs PBA. Data are means ± SD.

DISCUSSION

Whole body beta-alanine retention.

The main finding of study A is that of the total amount of chronically ingested SRBA (168 gr over 5 week period) only very little (~2.7 g or 1.6%) is excreted in the urine (figure 2). This is surprising because it could be calculated from previous studies (Harris 2006; Baguet 2009; Stellingwerff 2012) that muscular uptake and the incorporation efficiency of exogenous BA into carnosine are very low as well. In the present study, the amount of BA converted into muscle carnosine was calculated to be ~4.1g (2.8%), when assuming that 40% of body mass is muscle mass. Thus, it seems that the vast majority (~160g or 95-96%) of the ingested BA is neither going into muscle carnosine nor into the urine. Therefore, the alternative metabolic fate is currently unknown and merits further investigation. These new findings for BA are in sharp contrast with creatine, another popular ergogenic nutritional supplement. As for BA, it is a small nitrogen-containing, non-proteinogenic molecule with high abundance in skeletal muscle and a similar transsarcolemmal transport system. It has been repeatedly demonstrated, already upon the first publication in 1992 by Harris et al. (1992), that the major portion of the ingested creatine is either excreted in the urine or accumulated in the muscle, with a reciprocal relationship between the two options (Green 1996a; Green 1996b; Steenge 1998; Steenge 2000). It is clear that for BA, urinary excretion cannot be used to make an indirect estimation of muscular accumulation of carnosine.

This finding brings to mind an important question: where does all the BA go? One possibility is oxidation and energy provision. The contribution of alpha-amino acids to energy delivery in muscle and other tissues is usually quite low, as compared to carbohydrates and fat. However, when certain amino acids are available in excess, e.g. branched chain α-amino acid supplementation, then they will be oxidized and their contribution to total energy delivery can amount to up to 10% (Jeukendrup A. 2010). Indications exist that not only α-amino acids, but beta-amino acids as well can serve as an energy source when available in excess, which would certainly be the case with the ingestion of BA in such high daily doses (3.2 to 6.4 g/day; (Harris 2006; Hill 2007b; Baguet 2009; Stellingwerff 2012)). In order to enter the citric acid cycle the amino group of BA

has to be removed through a transamination reaction, catalyzed by BA transaminase (EC:2.6.1.19; also known as GABA transaminase (Griffith 1986; De Biase 1995)). Interestingly, we recently demonstrated its expression in rodent skeletal muscle and showed that its transcription is upregulated upon BA supplementation (Everaert 2013). Therefore, we suggest that BA oxidation could be an important metabolic fate of chronically ingested BA, possibly in skeletal muscle, but likely in other organs such as liver, kidney and brain (De Biase 1995; Ito 2001). However, it is not excluded that other metabolic pathways are involved as well. Yet, it seems unlikely that the conversion of BA into carnosine in other (non-muscle) tissues is of any quantitative significance, because presence of carnosine (Flancbaum 1990; Jackson 1996) and carnosine synthase (Harding 1976) in non-muscle tissues is several orders of magnitude lower than in muscle.

The effect of macronutrients on beta-alanine retention.

We observed that co-ingestion of carbohydrates and proteins (61 g CHO + 22 g P) with SRBA did not alter urinary BA excretion and hence BA retention (figure 2). However, as mentioned above, urinary BA excretion is not a good inverse estimate of muscle carnosine accumulation. Thus, this observation does not exclude a shift towards more muscle carnosine loading, if this depends on the insulin sensitivity of the sodium-dependent BA transporter. In order to resolve this question, we needed to look at muscle carnosine loading in a long-term protocol in which BA was ingested either in the presence or absence of hyperinsulinemia.

The effect of meal-timing on muscle carnosine loading.

In study B, we could confirm in a one-day pre-test that subjects taking PBA in between the meals and subjects taking PBA at start of the meals had different (respectively out-of-phase versus in-phase) blood profiles concerning plasma insulin and BA (figure 3). Respectively 82% and 0% of the total AUC of plasma BA showed elevated BA levels (> 30 µM) at the moment of insulinemia (> 25µU/ml). In the follow-up chronic supplementation study, we were able to demonstrate that carnosine loading in the insulin-sensitive soleus muscle increased by 64% when PBA (4 x 800 mg) is taken at the start of each meal/snack compared to 41% when PBA was ingested in between the meals (figure 4). This finding may have practical applications, i.e. regarding the advice on how and when to ingest BA

for optimal muscle carnosine loading results. Yet, it may also provide further insight into the physiological mechanism and limitations to muscle BA uptake and carnosine synthesis.

Most researchers postulate that very high physiological insulin levels (approaching ~ 100 µU/ml) are necessary to stimulate the equally sodium dependent transporters of creatine (CreaT) and carnitine (OCTN2) and hence to alter their retention and muscle concentration (Green 1996a; Green 1996b; Steenge 1998; Steenge 2000; Stephens 2007b). However, Greenwood et al. (2003) suggested that creatine retention can be increased even with relatively small amounts (18 g) of simultaneous carbohydrate ingestion. We could demonstrate that meal-timing alone can beneficially influence muscle carnosine loading. In figure 3, average meal-induced insulin plasma peaks are shown, but individual peaks vary between 53 and 102 µU/ml. Clausen et al. (2003) showed that Na+/K+ pumps in skeletal muscle are stimulated by insulin over a range of concentrations down to low physiological levels. Therefore, it is possible that meal-induced elevations in serum insulin are capable of triggering the sodium-dependent transporter TauT. In our study, a meal-induced effect could only be confirmed in soleus muscle. Considering the fact that this is a more insulin-sensitive muscle (James 1985), it is not unlikely that the soleus responds better on insulin induced BA uptake. In addition Lavoie et al. (1996) demonstrated that insulin-induced translocation of Na+/K+ ATPase subunits to the plasma membrane, one of the two possibilities to increase Na+/K+ ATPase activity, is restricted to oxidative fiber-type skeletal muscles which are predominantly present in soleus. However, caution remains warranted as other postprandial changes than an increase in serum insulin might be at play. In addition, one could argue that the the difference in dietary beta-alanine intake (PBA: 213 mg/day vs PBA+meal: 250 mg/day) is responsible for the difference in soleus carnosine loading (PBA: 41% and PBA+meal: 64%). However, dietary habits do not seem to influence muscle carnosine, as our lab (Baguet et al., unpublished results) could recently demonstrate that switching from an omnivorous diet to a vegetarian diet for 6 months does not after baseline muscle carnosine concentration.

Slow release versus pure beta-alanine supplementation.

To our knowledge, this is the first study that directly compares the loading efficiency of a similar dose of PBA and SRBA in a chronic supplementation study. It was initially hypothesized that SRBA would be more efficient than PBA because of a better BA retention (resp. 98.9% and 96.3%) upon acute ingestion (Decombaz 2012). We now know that whole BA retention is not a good indicator of muscle carnosine loading efficiency. In the current study, we could show that there was no significant difference between long-term supplementation of SRBA (SRBA+meal) compared to PBA (PBA+meal) (figure 4B). If we believe that the percentage of the AUC of plasma BA, that occurs during hyperinsulinemia, is of importance for enhancing BA uptake in skeletal muscle, we may expect that 'SRBA+meal' is more responsive than 'PBA', but less than 'PBA+meal'. This expected order is observed in the soleus, but not in the gastrocnemius. Possibly, this is merely an epiphenomenon and also other aspects of the kinetics, such as the peak plasma BA concentration and the total AUC for BA may play a role in the effectiveness of BA on muscle carnosine loading.

Conclusion

In this article we show that despite only a small portion of chronically ingested BA is incorporated into muscle carnosine, there is equally only a very small portion lost through urinary excretion. We therefore hypothesize that the majority of ingested BA is metabolized. Secondly, we show that BA supplementation is more effective when co-ingested with a meal, suggesting that insulin could play a role in muscle carnosine loading. Finally, we conclude that SRBA is equally effective as PBA in muscle carnosine loading.

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Study 2

THE BETA-ALANINE DOSE FOR MAINTAINING MODERATELY ELEVATED MUSCLE CARNOSINE LEVELS

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ABSTRACT

Introduction. Chronic beta-alanine (BA) supplementation is an increasingly popular nutritional strategy, because it can elevate muscle carnosine content and thereby enhance high-intensity exercise performance. The current study investigates 1) whether sex and body mass are determinants of BA induced muscle carnosine loading, and 2) the optimal maintenance dose for ensuring constantly elevated muscle carnosine stores.

Methods. During the loading phase, 34 participants (men and women) were supplemented with 3.2g (4x800 mg) BA/day for 46 days (slightly different loading strategies were applied concerning the effect of meal-timing and supplementation form (Stegen et al. 2013)). Thereafter, 19 participants (men and women) continued taking free-powder BA for 6 more weeks (maintenance phase). The participants were matched and re-divided into three groups respectively receiving 0.4g BA/day, 0.8g BA/day and 1.2g BA/day. Muscle carnosine content was measured in m. soleus and m. gastrocnemius using ^{1}H-MRS.

Results. Body mass and sex had only minimal effect on the absolute increase in muscle carnosine. Given the lower baseline values of women, the relative increase for women was higher, indicating that women require less BA for the same relative increase. In addition, a significant negative correlation was observed between body mass and the relative increase in muscle carnosine ($r=-0.45$, $p=0.007$). A maintenance dose of ~1.2 g BA/day, was most effective to keep muscle carnosine content elevated at post-supplementation level.

Conclusion. Sex and body mass do not markedly affect the absolute increase during muscle carnosine loading, although they are determinants for the relative increase. Additionally, we established for the first time an effective maintenance dose of ~1.2g BA/day to keep muscle carnosine content elevated at 30-50% above baseline for a prolonged period.

Keywords: nutritional supplements, histidine-containing dipeptides, buffering capacity, exercise performance, carnosine, beta-alanine

INTRODUCTION

Beta-alanine is an increasingly popular nutritional supplement. It is the rate-limiting precursor for carnosine synthesis, a cytoplasmic dipeptide (β-alanyl-L-histidine) in human skeletal muscle (Harris 2006). Increasing muscle carnosine is associated with performance enhancement in high-intensity exercise (Derave 2007; Hill 2007a; Baguet 2010; del Favero 2012). The ergogenic mechanism of carnosine presumably relates to its characteristics as a calcium sensitizer (Dutka 2012; Everaert 2012) and proton buffer (Parkhouse 1985; Baguet 2011). However, various other research fields have investigated this molecule for other biological qualities such as scavenger of reactive oxygen species, reactive nitrogen species and deleterious aldehydes (malondialdehyde, methylglyoxal, hydroxynonenal, …), chelator of zinc and copper ions, and antiglycating and anticross-linking activities (Aldini 2005; Boldyrev 2012; Boldyrev 2013). These properties suggest a therapeutic potential of muscle carnosine loading in clinical populations, a research topic beginning to emerge (del Favero 2012; Gualano 2012; Sale 2013).

An efficient regimen to augment human muscle carnosine content was initially established by Harris and colleagues (Harris 2006). The latter and subsequent studies have shown that chronic oral supplementation of 1.6 to 6.4g/day of beta-alanine will generally lead to increases in muscle carnosine content of ~15-85% in 4 to 12 weeks (Hill 2007b; Baguet 2009; del Favero 2012; Stellingwerff 2012). Even though we may not yet have attained the true maximal or optimal loading protocol, supplementation strategies aiming for moderately elevated muscle carnosine levels are frequently used in sports and have proven ergogenic effects (Derave 2007; Hill 2007b; Baguet 2010). In contrast, not a single study has so far established a suitable maintenance dose. This gap hampers long-term studies on effects of more chronically elevated carnosine levels, which is more relevant in clinical situations and aging. The current study focuses on establishing a suitable maintenance dose, although for a specific loading protocol (46 d of supplementation with 3.2 g/d of beta-alanine).

It is known that upon discontinuation, muscle carnosine levels will gradually return to baseline (pre-supplementation) levels in 6-20 weeks (Baguet 2009; Stellingwerff 2012). A first attempt to establish such maintenance dose for beta-alanine supplementation was

published by Stellingwerff et al. (2012). They demonstrated that after an initial increase achieved by a 4 week loading protocol of 3.2g BA/day, a further ~30% increase in muscle carnosine occurred after 4 weeks of taking 1.6g beta-alanine/day. We can conclude that the optimal dose for maintaining elevated muscle carnosine stores must be found between 0 and 1.6g BA/day. Therefore, we will investigate 3 intermediate doses: 0.4g/day, 0.8g/day and 1.2g/day.

Little is known about the influence of gender, body mass and fat-free mass (FFM) on muscle carnosine loading via BA supplementation in the current literature. Previously published reports on the effects of beta-alanine supplementation on human muscle carnosine content included exclusively male participants (Harris 2006; Derave 2007; Hill 2007b; Baguet 2009; Baguet 2010; Stellingwerff 2012; del Favero 2012). Only one study (del Favero 2012) included both elderly men and women, but they didn't diversify between sexes. Some studies have reported positive effects of beta-alanine supplementation on exercise performance in women (Stout 2007; Stout 2008), yet without measurement of muscle carnosine content. In addition, it has been shown that carnosine metabolism becomes gender-specific in humans upon sexual maturation (Everaert 2010; Baguet 2012), with muscle carnosine content being lower and serum carnosinase activity being higher in adult women versus men. Several reasons for this sexual dimorphism have been postulated, such as muscle fiber type distribution or diet, but animal experiments have shown that testosterone is certainly involved (Penafiel 2004; Everaert 2013). Up until now, it was not clear whether the factors inducing differences in baseline muscle carnosine content would also result in differences in carnosine loading between men and women. Therefore, we now aim to establish whether women respond equally well, better or worse to BA supplementation.

Since women are in general lighter and have less FFM, we could assume a larger muscle carnosine loading because they receive a higher dose per kilogram body mass or FFM. In comparison with other nutritional supplements having ergogenic effects during high intensity exercise, the recommended dosing protocol is either a fixed amount irrespective of body mass (creatine (Harris 1992; Hultman 1996)) or body mass corrected (sodium bicarbonate and sodium citrate (Van Montfoort 2004)). However, it remains to be established whether BA should be dosed in relation to body mass.

In summary, this study was designed in order to define the optimal maintenance dose for ensuring constantly elevated muscle carnosine stores of 30-50% above baseline, secondly to clarify whether sex and body mass are influencing factors during muscle carnosine loading and finally to provide an explanatory model of loading, maintenance and washout of elevated muscle carnosine stores.

METHODOLOGY

Participants

Thirty-four participants (men and women) volunteered to participate in this study. All participants were healthy, non-vegetarian and physically active, but not involved in regular training. The study (loading and maintenance phase) was approved by the local ethical committee (Ghent University Hospital, Ghent, Belgium), all participants gave their written informed consent to take part in the study and were aware that they were free to withdraw from the experiment at any point.

Study design

During the *loading phase,* 34 participants (16 men: age: 19.4 ± 1.1 yr, body mass: 73.9 ± 8.2 and 18 women: age: 19.3 ± 0.9, body mass: 60.2 ± 6.0) were supplemented with 3.2 g (4x800 mg) of beta-alanine per day for 6 weeks (46 days). These data were collected from a previous study with another research question (Stegen 2013), where all participants were randomized into three gender- and body mass-balanced groups (group 1 received free-powder beta-alanine between the meals, group 2 received free-powder beta-alanine in between the meals and group 3 received slow-release beta-alanine). Although the three groups were slightly different loaded, it allowed us to use this dataset to study the effect of body mass (BW) , fat-free mass (FFM) and gender on muscle carnosine loading, since participants with different body mass and gender were equally dispersed among the three groups (group 1: BW: 55-84kg, FFM: 43-72kg; group 2: BW: 54-85kg, FFM: 45-81 and group 3: BW: 49-88, FFM: 37-61). Group 1 and 2 received CarnoSyn™ powder in capsules and group 3 CarnoSyn™ slow-release (SR) tablets, both delivered by Natural Alternatives International (NAI) and analysed using documented HFL screening method by HFL sport science. Muscle carnosine content was measured before and after the loading phase. Therefore, the percentage of ingested beta-alanine accumulated in skeletal muscles could be calculated (vide infra). Anthropometrical variables (length, body mass, fat-free mass) and dietary beta-alanine ingestion were also assessed at the start of the supplementation period.

During the *maintenance phase*, 19 participants volunteered to continue taking free-powder beta-alanine for 6 weeks in order to determine *a maintenance dose*. The

participants (12 men: age: 19.8 ± 1.1 yr, body mass: 74.5 ± 8.1 kg and 7 women: age: 19.7 ± 1.0, body mass: 60.1 ± 4.3 kg) were re-divided into three groups respectively receiving 0.4g /day, 0.8g /day and 1.2g /day of free-powder BA (CarnoSyn^{TM} powder, respectively 1, 2 or 3 capsules dispersed among the day (before the meal)). Following loading, and because of the high drop-out, the participants were re-stratified for 1) loading history (carnosine content post-loading, absolute and relative increase in muscle carnosine during the loading phase), 2) body mass and 3) gender in order to obtain three new and matched groups for the maintenance phase. Muscle carnosine was measured again on the end of the 6-week maintenance dose.

Determination of muscle carnosine content

Carnosine content of all the participants was measured by proton magnetic resonance spectroscopy (^{1}H-MRS) in soleus and gastrocnemius medialis muscles, as described by Baguet et al. (2010). When both muscles respond similarly, the average of the two muscles is taken and referred to as 'muscle carnosine'. Otherwise, the soleus or the gastrocnemius muscle is mentioned separately. The participants were lying in supine position on their back and the lower leg was fixed in a holder with the angle of the ankle at 20° plantar flexion. All the MRS measurements were performed on a 3-T 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) of 2.000 ms, echo time (TE) of 30 ms, number of excitations is 128, 1.024 data points, spectral bandwidth of 1.200 Hz, and a total acquisition time of 4.24 min. The average voxel size for the soleus and gastrocnemius muscle was respectively 40 mm x 10 mm x 28 mm and 40 mm x 11 mm x 30 mm. The line width of the water signal for the soleus and gastrocnemius muscle was on average respectively 24.6 Hz and 27.6 Hz, following shimming procedures. The absolute carnosine content (in millimolar; mM) was calculated as described before by Baguet et al. (2010) and they reported a relatively low coefficient of variation of 9.8% in soleus and 14.2% in gastrocnemius (Baguet 2009).

Daily dietary BA intake and % BA accumulated in skeletal muscles

An estimation of the daily dietary beta-alanine ingestion was achieved by a 2- week record of their meat and fish consumption, as described by Baguet et al. (2009). During the loading phase the percentage of the total ingested amount of beta-alanine (147 g) that is accumulated into the skeletal muscles is estimated for every subject separately by assuming that 46% of body mass is muscle mass for 19-years-old men and 33% for 19-years-old women (Janssen 2000) and by taking into account their personal average increase in muscle carnosine.

Anthropometry

Height and body mass were determined using a digital balance scale and a stadiometer. Fat-free mass (FFM) was assessed by bio-impedance (Bodystat 1500 MDD; Bodystat Ltd., Douglas, Isle of Man, UK). For the latter, only a subgroup (n= 23; group 1: 2/3; group 2: 4/4; group 3: 3/5; men/women) was measured because the equipment (Bodystat 1500MDD) was not always available. Patients were in supine position for at least 5 min. Surface electrodes were attached to the right hand (red lead: behind the knuckle of the middle finger; black lead: on the wrist to the ulnar head) and foot (red lead: behind the second toe next to the big toe; black lead: on the ankle at the level of and between the medial and lateral malleoli). FFM was derived from the impedance and the deducted total body water. The intraclass correlation coefficient (ICC) for FFM-measurements with the Bodystat 1500 MDD, is 0.987 (Steiner 2002).

Statistics

All correlations were evaluated by Pearson correlations. An independent two-sample t-test was used to study gender differences during loading. To examine whether our three maintenance dose groups evolved differently in time, we performed a repeated measures analysis of variance (general linear model) with ‘group’ (0.4g/day, 0.8g/day and 1.2g/day) as between-participants factor and ‘time’ (post-loading and post-maintenance) as a within-participants factor. In case of significant interaction and to determine whether a specific maintenance dose results in a decrease/increase compared to post-loading a paired sample t-test was performed for each group separately. All analyses were done

with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p < 0.05$.

RESULTS

Effects of sex , body mass and fat-free mass on the loading phase.

The absolute increase in muscle carnosine, after taking 3.2g BA/day for 6 weeks, was approximately 1.5-2 mM and was equal irrespective of the *muscle type* and *gender* (figure 1A). Logically, since we confirm that the soleus muscle has lower baseline values compared to the gastrocnemius (SOL: 3.47 ± 0.87mM , GAS: 4.51 ± 1.36mM, $p<0.05$), the relative increase in soleus is higher (SOL: + 53.4 ± 30.1%, GAS: + 41.8 ± 26.8%). Similarly, women have lower baseline carnosine values compared to men (women: 3.28 ± 0.58 mM , men: 4.79 ± 0.85 mM , $p<0.05$), therefore the relative increase in women is significantly higher in gastrocnemius compared to men (women: + 50.8 ± 30.7%, men: +31.6 ± 17.5 %) however, not for soleus (figure 1B). The percentage of the ingested beta-alanine that is accumulated in the skeletal muscles is higher in men (3.6 ± 1.1%) compared to women (1.9 ± 0.8%; $p<0.001$), however this is explained by the higher body mass and muscle mass in men compared to women (body mass: men: 73.9 ± 8.2kg; women: 60.2 ± 6.0kg, $p<0.001$; muscle mass: men: 34.0 ± 3.7 kg; women: 19.6 ± 2.0 kg, $p<0.001$).

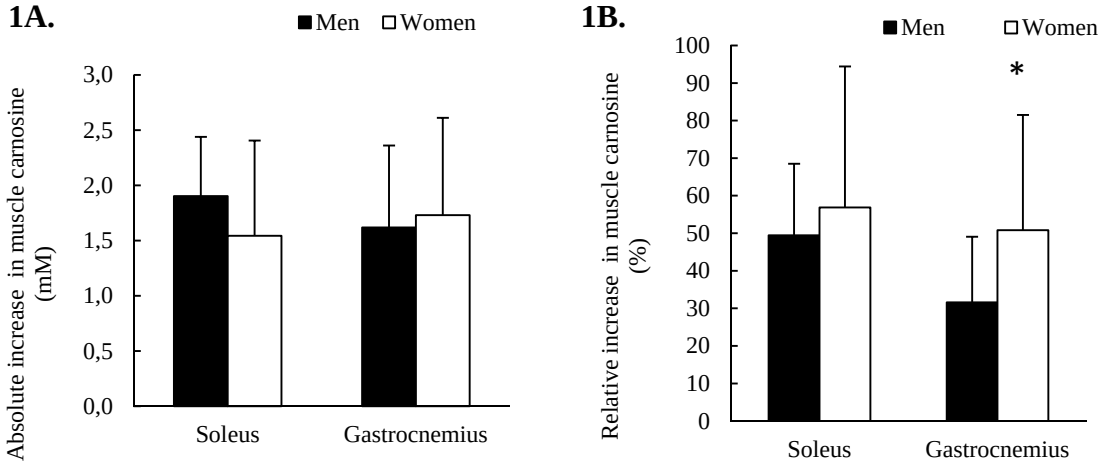

Figure 1: Loading phase: Comparison between sexes for absolute (1A) and relative (1B) increase in muscle carnosine after a supplementation period of 3.2 g BA/day during 46 days . Data are means ± SD. * significant for $p < 0.05$ (women vs men).

In order to assess the effects of *body mass* and *fat-free mass (FFM)* on muscle carnosine loading, correlations were made with the absolute and relative increase in muscle carnosine, separately for gender and muscle type (figure 2). Heavier men have a lower absolute increase in soleus carnosine compared to lighter men (r=-0.51, p=0.044, figure 2A). Nevertheless, this finding could not be confirmed for women and gastrocnemius muscle (figure 2A and 2B). In addition, correlations between body mass and the absolute increase in muscle carnosine are not stronger when taking FFM into account. For the relative increase in muscle carnosine, a stronger relationship with body mass is present (r=-0.45, p=0.007 for muscles and sexes pooled), which remains predominantly present for men (SOL: r=-0.53, p=0.036; GAS: r=-0.47, p=0.065), and not for women (SOL: r=-0.10, p=0.699; GAS: r=-0.44, p=0.069) (figures 2C and 2D).

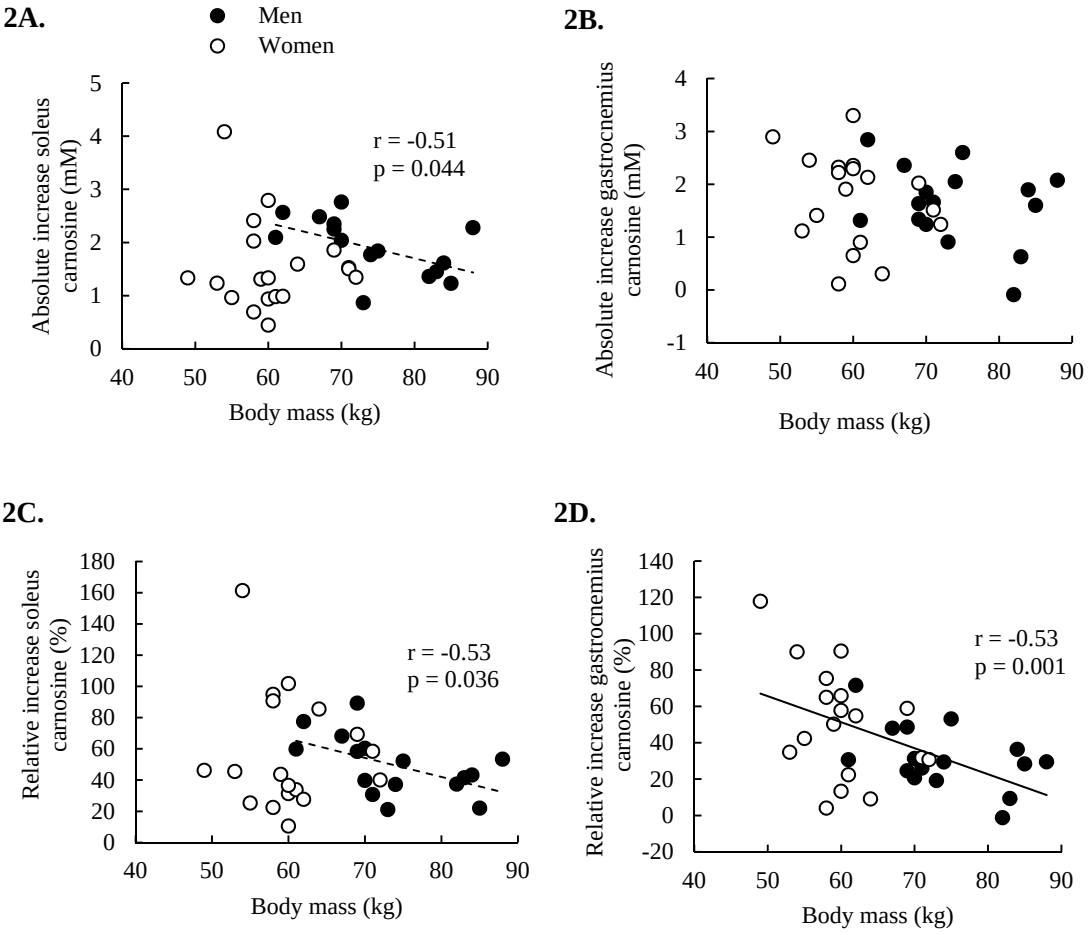

Figure 2: Loading phase: Correlation between body mass and the absolute (2A: soleus, 2B: gastrocnemius) and relative (2C: soleus and 2D: gastrocnemius) increase in muscle carnosine for a supplementation protocol of 3.2g BA/day for 46 days. White and black circles represent respectively women and men. Dashed and solid lines represent significant correlations for respectively men only and the entire group. Correlations are significant when $p<0.05$.

Finally, *baseline carnosine* content did not influence the absolute increase in muscle carnosine (men: r=-0.23, p=0.222; women: r=-0.28, p=0.264). As a consequence, men and women with higher baseline values have a lower relative increase compared to those with lower baseline values (men: r=-0.74, p=0.001; women: r=-0.56, p=0.015).

Maintenance dose

No significant differences were present between groups prior to the start of the maintenance phase concerning subject characteristics and loading history of muscle carnosine (table 1). The group that took 1.2g BA/day remained at the post-loaded level, while the other two groups (0.4g BA/day and 0.8g BA/day) showed a decrease relative to the initial increase in muscle carnosine with respectively -46% and -32% (figure 3). In addition, a strong linear relationship is present (r=0.71; p=0.001) between the maintenance dose, corrected for body mass (mg/kg BW per day), and the maintenance in muscle carnosine (with 100% indicating perfect maintenance and 0% indicating complete return to baseline). From this relationship, optimal maintenance dose is calculated at ~18 mg/kg BW/day.

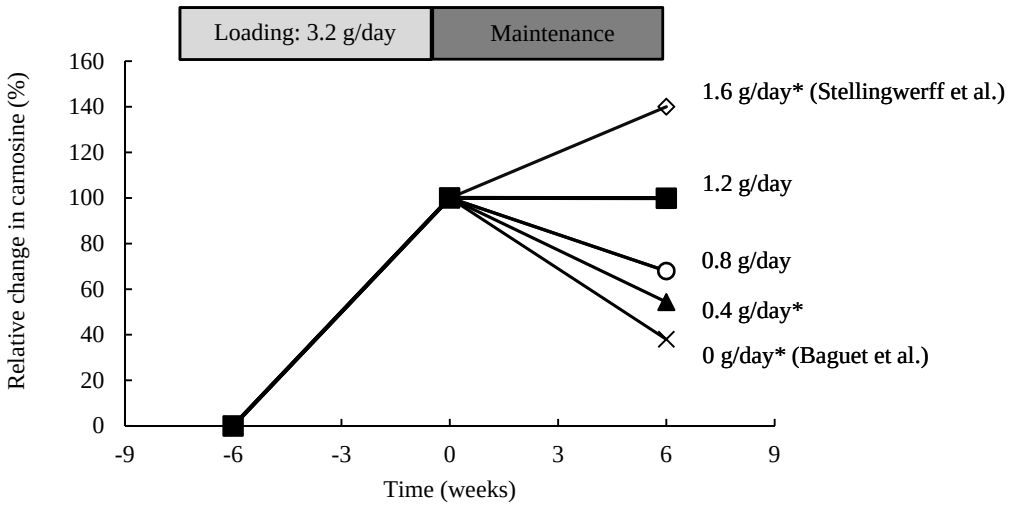

Figure 3 : Maintenance phase: Five different maintenance doses on a time scale are shown: before loading (0%), after 6 weeks loading with 3.2g BA/day (100%) and after 6 weeks receiving a maintenance dose: 1.2g/day (n=7), 0.8g/day (n=6), 0.4g/day (n=6). The post-maintenance doses are relative to the initial increase during loading. Data of 0 and 1.6g/day are derived from Baguet et al. (Baguet 2009) and Stellingwerff et al. (Stellingwerff 2012) respectively. * $p < 0.05$ different from post-loading.

		0.4 g/day	0.8 g/day	1.2 g/day
Participants characteristics				
Participants (n, M/F)		7 (4/3)	6 (4/2)	6 (4/2)
Age (yr)		19.6 (± 1.0)	20.3 (± 1.0)	19.3 (± 0.8)
Body mass (kg)		67.0 (± 11.4)	68.7 (± 8.6)	72.3 (± 10.1)
Dietary beta-alanine intake (mg/day)		267 (± 81)	318 (± 194)	243 (± 66)
Loading history				
Muscle carnosine concentration	SOL	3.63 (± 0.57)	3.73 (± 0.75)	3.79 (± 1.16)
pre-loading (mM)	GAS	4.64 (± 1.28)	4.92 (± 1.64)	5.14 (± 1.35)
Muscle carnosine concentration	SOL	5.31 (± 0.68) [a]	5.14 (± 1.01) [a]	5.78 (± 1.14) [a]
post-loading (mM)	GAS	6.72 (± 1.69) [a]	6.43 (± 1.18) [a]	6.66 (± 0.81) [a]
Absolute increase in muscle carnosine	SOL	1.68 (± 0.79)	1.41 (± 0.58)	1.99 (± 0.55)
during loading (mM)	GAS	2.07 (± 0.92)	1.52 (± 0.95)	1.52 (± 0.59)
Post-maintenance carnosine concentration				
Muscle carnosine concentration	SOL	4.50 (± 0.68) [a, b]	4.71 (± 1.27) [a, b*]	5.71 (± 0.69) [a]
post-maintenance (mM)	GAS	6.00 (± 1.38) [a, b]	6.19 (± 1.48) [a]	6.83 (± 0.71) [a]

Table 1: Maintenance phase: subject characteristics, muscle carnosine loading history and post-maintenance carnosine concentration for the three groups (0.4g BA/day, 0.8g BA/day and 1.2g BA/day as maintenance dose). SOL and GAS are respectively soleus and gastrocnemius. a $p<0.05$ vs preloading, b $p< 0.05$ vs postloading, b* $p<0.1$ vs postloading. Data are means ± SD.

DISCUSSION

The current study further examined possible determinants of beta-alanine induced muscle carnosine loading and newly established an efficient maintenance dose, following a 46-day supplementation protocol of 3.2g beta-alanine per day.

This study shows that sex and body mass have only minimal effect on the absolute increase during muscle carnosine loading. It has already been shown that soleus and gastrocnemius muscle respond equally well to beta-alanine supplementation in men, irrespective the higher absolute carnosine baseline value of the gastrocnemius. This study confirms similar absolute increases of carnosine in the gastrocnemius and soleus muscles in females, suggesting that there are no muscle type or sex differences in the response to beta-alanine supplementation. However, it remains a subject of debate whether the higher relative increases in women (figure 1B) or lighter people (figures 2C and 2D) are relevant from a practical point of view. It has already been reported that there is a correlation between the relative change in carnosine and performance enhancement (Hill 2007b; del Favero 2012). In addition, Baguet et al. (2010) (data from our own lab), published the correlation between the absolute increase in muscle carnosine and the absolute improvement on a 2000m rowing test in highly trained rowers. The manuscript only reported the absolute values, but the correlation remained present when values were expressed relatively (data not published: $p=0.024$, $r=-0.543$). Nevertheless, it remains questionable whether this is an argument for establishing a model where gain in performance is primarily related to the relative change in carnosine. In figure 4, we have graphically demonstrated the relevance of body mass and sex for increasing muscle carnosine content with 50%. Although this increase in muscle carnosine is probably not the most optimal increase for the most optimal improvement in performance, it is considered effective for ergogenic and function-enhancing purposes.

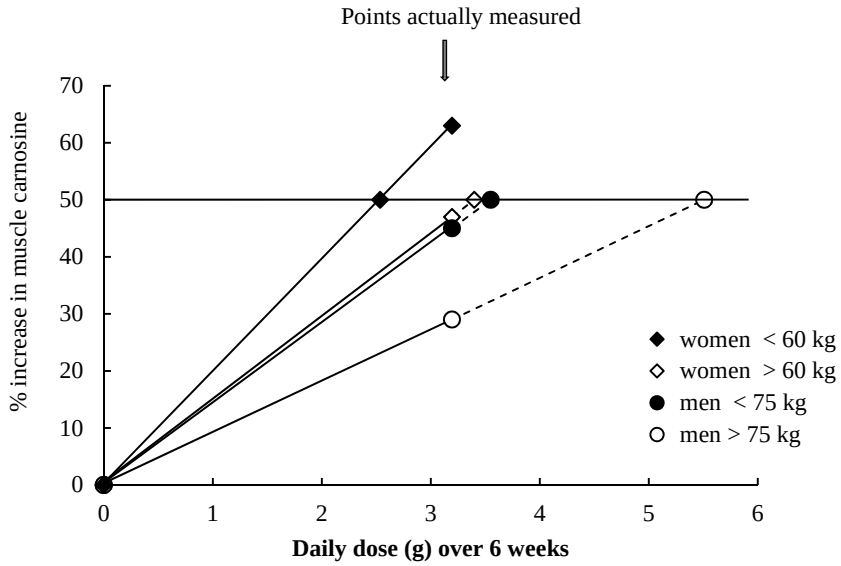

Figure 4: Loading phase: The relationship between the daily BA dose (3.2g/day for the entire group) and the relative increase in muscle carnosine for lighter (< 60 kg) and heavier (> 60 kg) women (diamonds), and for lighter (< 75 kg) and heavier (> 75 kg) men (circles). Dashed lines are the extrapolations to reach a 50% increase in muscle carnosine.

A nicely performed meta-analysis (Hobson 2012), covering 18 studies, about the effect of beta-alanine supplementation on exercise performance clearly demonstrated an ergogenic effect in exercise lasting 60-240s. However, there are only 4 studies that measured muscle carnosine as well. These studies reported increases in muscle carnosine with +37% (Baguet 2010), +42% (Derave 2007), +59% (Hill 2007b), +85% (del Favero 2012), averaging ~50%. So, in figure 4, we have extrapolated (or intrapolated) the daily dose that would be required to achieve a 50% increase over a 6 week loading phase in the men and women, each divided in a lighter and heavier half, with a cut-off value of 60 kg for the women and 75 kg for the men. This post-hoc calculation indicates that the female participants weighing less than 60 kg, with an average of 56 kg, should ingest 2.5 g/day, whereas heavier women (~ 64 kg) and lighter men (~ 69 kg) should ingest respectively 3.4 g/day and 3.6 g/day and heavier men (~ 84 kg) 5.5 g/day (figure 4). If faster loading is aimed, BA dose can be increased up to 6.4 g per day with serving doses of 800mg per intake (or 1600 mg in case of slow-release beta-alanine). Higher doses may not be advised at present because the effectiveness or side-effects have not been studied beyond this dose.

The present study aimed at determining effective maintenance dose. Previous studies (Baguet 2009; Stellingwerff 2012) have shown that following muscle carnosine loading, a slow wash-out takes place ultimately leading to return to baseline values. This indicates there is some kind of elimination process. However, the nature of the elimination process is still unclear at the moment: it could be either 1) an active release through outward-directed transarcolemmal transporters (e.g. through PHT1 or PHT2 (Everaert 2013a)), 2) a passive loss of carnosine through sarcolemmal disrupture (Dunnett 2002), 3) an enzymatic degradation (hydrolysis into beta-alanine and L-histidine) through carnosinase (CN2, (Everaert 2013)), 4) a combination of these. The carnosine elimination rate is most likely positively related to the muscle carnosine content itself, namely the more you have, the more you can lose or degrade. Net muscle carnosine turnover is a balance between the carnosine synthesis and elimination rate and an effective maintenance dose would be one where the synthesis equals the elimination. The carnosine synthesis rate in muscle is mainly determined by the sarcoplasmic beta-alanine availability, as beta-alanine is the rate-limiting precursor. Sarcoplasmic beta-alanine availability is largely determined by dietary beta-alanine intake (Harris 2006) and the enzymatic synthesis and degradation pathways of beta-alanine.

In figure 5, we have graphically displayed a hypothesis for the alterations in net carnosine turnover rate in different situations (baseline, loading, washout and maintenance) as a result of changes in carnosine synthesis and/or elimination rate. At baseline, there is no net change in muscle carnosine as synthesis and elimination are in equilibrium and both at a low level. During beta-alanine supplementation (loading), carnosine synthesis will markedly increase because of increased precursor availability and net turnover will be strongly positive. As supplementation continues, with long duration and high doses, the elimination rate will gradually increase as muscle carnosine content and thus elimination becomes higher. As a result, the net positive turnover rate will become smaller, as has been demonstrated by Hill et al. (2007b), where carnosine loading efficiency was much smaller in the second half (+20%) compared to the first half (+60%) of supplementation. Upon discontinuation of supplementation, elimination rate is still elevated, whereas synthesis rate falls back to baseline values, resulting in a net negative balance, causing wash-out (Baguet 2009; Stellingwerff 2012). Finally, keeping muscle carnosine at a

constant elevated level (maintenance) requires that carnosine synthesis rate is increased by a low dose of beta-alanine supplementation, in order to compensate for the increased carnosine degradation rate in carnosine-loaded muscles.

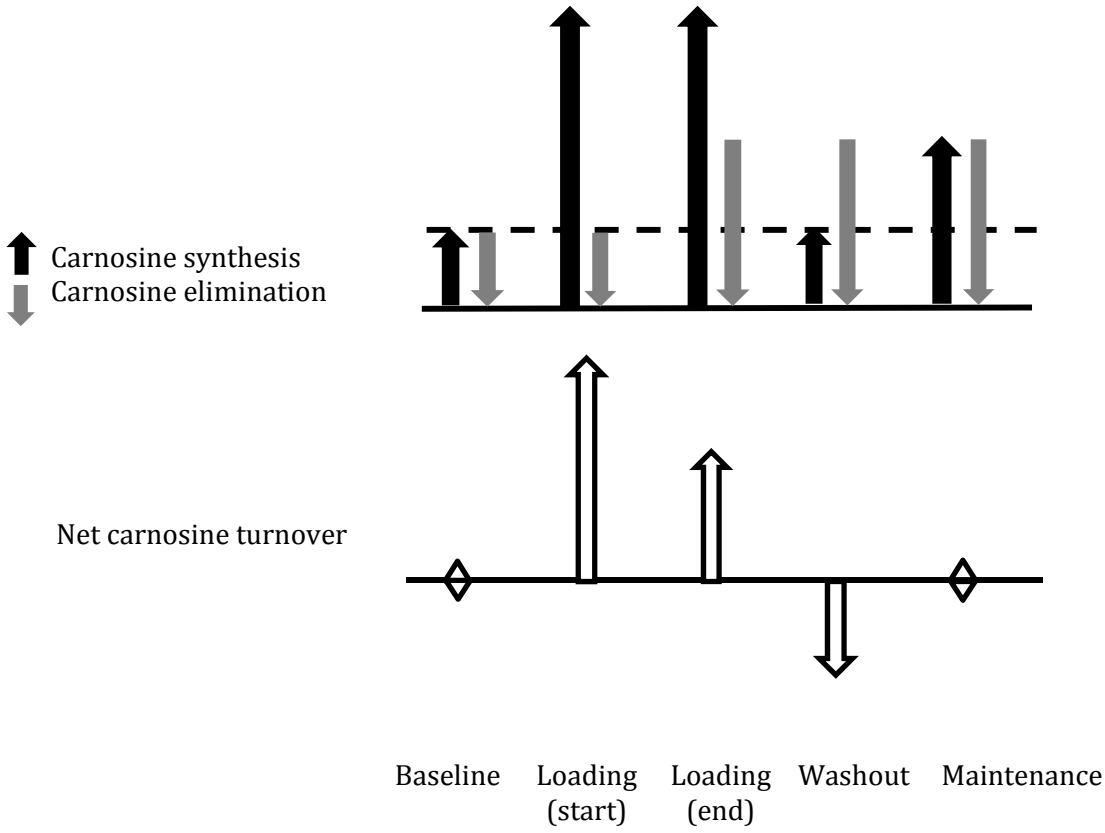

Figure 5: Graphical representation of a hypothesis on muscle carnosine turnover, which could explain the currently observed effects of the different phases of beta-alanine supplementation on muscle carnosine content**.**

We now determined that an average dose of 1.2 g/day is considered optimal to keep muscle carnosine content elevated at 30-50% above baseline. Similar with the loading phase, it seems that heavier participants need a higher maintenance dose to keep muscle carnosine levels at 100% loaded, compared to more lighter participants, since there is a strong relationship between maintenance dose, corrected for body mass, and maintenance in muscle carnosine. Therefore, we suggest taking ~18 mg/kg BW/day to keep up elevated muscle carnosine levels. However, as the carnosine degradation rate is positively related to the muscle carnosine content, it can be hypothesized that the required maintenance dose may be higher to keep muscle carnosine elevated at supra-

maximal levels (>100% or even higher above baseline). Future studies will have to investigate this issue.

In conclusion, sex and body mass are not major determinants for the absolute increase in muscle carnosine during loading, although they can partly explain the variability in the individual response in muscle carnosine loading when expressed as percentual increase versus baseline. Furthermore, the establishment of a suitable maintenance dose (~1.2g BA/day), following a supplementation period of 3.2g BA/day, will now allow for studying the effects of chronically elevated muscle carnosine stores in situations where this is required, such as prolonged sports competition period or sustained improvement of muscle functionality at old age (del Favero 2012).

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Study 3

PLASMA CARNOSINE, BUT NOT MUSCLE CARNOSINE, ATTENUATES HIGH-FAT DIET INDUCED METABOLIC STRESS.

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ABSTRACT

Introduction. There is growing in vivo evidence that the dipeptide carnosine has protective effects in diabetes and diabetes-related diseases. A critical unanswered question is its site of action, being either carnosine levels in tissues or plasma. This will be investigated using oral carnosine versus beta-alanine supplementation in a very mild high-fat diet rat model.

Methods. Thirty-six male Sprague-Dawley rats received either a control diet (CON), a 60% high-fat diet (HF), a HF-diet with 1.8 % carnosine (HFcar), or a HF-diet with 1% beta-alanine (HFba), as beta-alanine can increase muscle carnosine without an increase in plasma carnosine. Insulin sensitivity, inflammatory-signaling and lipidoxidative stress was determined in skeletal muscles and blood. In a pilot study, urine was collected.

Results. The three HF-groups were significantly heavier compared to CON. HFcar and HFba equally increased muscle carnosine concentrations and only HFcar reported elevated plasma carnosine levels and carnosine-HNE adducts. Elevated plasma and urine CML in HF was reduced by ~50% in HFcar, but not in HFba. Likewise, muscle iNOS mRNA is decreased by 47% ($p<0.05$) in HFcar, but not in HFba.

Conclusions. We can conclude that plasma carnosine, and not muscle carnosine, is involved in preventing early-stage lipidoxidation in circulation and muscle inflammatory signaling.

Keywords: Quenching ability carnosine, advanced lipidoxidation end-products, advanced glycation end-products, inflammatory signaling.

INTRODUCTION

All forms of diabetes are characterized by chronic hyperglycemia and are associated with (glyc)oxidative and low-grade inflammatory stress (Brownlee 2001; Stehouwer 2002; Mangge 2004; Brownlee 2005; Wellen 2005). The molecule carnosine, an endogenous histidine-containing dipeptide (β-alanine-L-histidine), is a potential candidate to counteract the development of diabetes and diabetes-related diseases. Beneficial effects of carnosine administration have been shown in both type 1 and type 2 diabetic rodent models (Lee 2005; Soliman 2007; Sauerhofer 2007). Additionally, carnosine was also found to be effective in reducing diabetes-related diseases, such as nephropathy and retinopathy in diabetic animal models (Pfister 2011; Riedl 2011; Aldini 2011). Current evidence in humans is still very scarce, but genetic studies in type-II diabetics suggest that polymorphisms leading to high carnosinase (CN1) expression, which impedes high circulating carnosine levels (Everaert 2012), are associated with increased risk of diabetic nephropathy (Janssen 2005; Mooyaart 2010).

The protective effect of carnosine could be related to a broad range of (bio)chemical properties, which is recently reviewed by Boldyrev et al. (2013). First, it may be related to a hypoglycemic effect (Yamano 2001; Nagai 2003; Lee 2005; Sauerhofer 2007; Soliman 2007), possibly by increasing plasma insulin, which in turn is evoked by the ability of carnosine to regulate the autonomic nervous system via the histamine H3 receptor (Yamano 2001; Nagai 2003), as well as to preserve or increase β-cells mass of the pancreas (Sauerhofer 2007). Second, carnosine has antioxidant activity in physiological conditions and induces sparing of (non)-enzymatic antioxidants (Lee 2005; Aydin 2010; Kim 2011). This is demonstrated by Lee et al. (2005), who found that decreased levels of catalase and glutathione peroxidase in liver and kidney of STZ-induced diabetic Balb/cA mice are increased by carnosine administration. Third, carnosine can scavenge reactive carbonyl species and reduce formation of advanced lipoxidation end-products (ALEs) and advanced glycoxidation end-products (AGEs). A study of Aldini et al. (2011) with Zucker obese rats demonstrated a protective/suppressive effect of carnosine on elevated protein carbonyls in kidney and elevated total AGEs and 4-hydroxy-2-nonenal-carnosine Michael adducts (CAR-HNE) in urine of the rats, which confirms the quenching ability of carnosine in vivo. Finally, carnosine appears to have anti-inflammatory characteristics,

demonstrated by its ability to decrease the expression of tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS) in striatum (Tsai 2010) and plasma (Lee 2005; Liu 2008) in mice.

A critical unanswered question regarding the potential therapeutic benefit of carnosine in metabolic disorders, is its site of action, being either the tissues or the plasma. The first possibility suggests that carnosine supplementation may be effective in correcting decreased levels of endogenous tissue carnosine, which have been demonstrated in diabetic kidney (Riedl 2011), retina (Pfister 2011) and skeletal muscle (Gualano 2012). Over 99% of total body carnosine is located in skeletal muscle (Boldyrev 2006; Derave 2007) and skeletal muscle is particularly important in the development of the insulin-resistant state (DeFronzo 2009; Abdul-Ghani 2010). If carnosine would perform its beneficial function in skeletal muscle, restoring or boosting endogenous muscle carnosine levels via carnosine administration could antagonize oxidation- and glycation-induced carbonylation and low-grade inflammation, which are characteristic of the insulin-resistant skeletal muscle (Oh-Ishi 2003; Bonnard 2008; Kewalramani 2010).The second possibility is that the reported positive effects of carnosine administration result from its action in the plasma (Aldini 2011) where it can scavenge cytotoxic compounds, such as reactive carbonyl species, and hence indirectly protect tissues. In order to distinguish between both possible mechanisms, beta-alanine, the rate-limiting precursor for carnosine synthesis (Bakardjiev 1994; Harris 2006), can be supplemented. It has been shown that beta-alanine supplementation increases muscle carnosine, with no concomitant increase in plasma carnosine (Harris 2006). Therefore, if the beneficial effects of carnosine administration can be replicated by beta-alanine administration, it means that the protective mechanism mainly related to restoring/elevating tissue carnosine levels. In vitro experiments have demonstrated that the biochemical properties of carnosine are predominantly related to the amino acid L-histidine (Kohen 1988; Egorov 1997; Decker 2000) or the imidazole moiety of L-histidine (Fontana 2002), or by the combination of the amino group of beta-alanine and the imidazolic ring of L-histidine (Aldini 2002; Liu 2003), but not by the amino-acid beta-alanine alone.

Clarifying the site of action of carnosine is particularly important for the translation of animal findings to patients. Raising tissue (muscle) carnosine concentrations in humans

has been found to be relatively easy by means of chronic oral beta-alanine administration (Harris 2006), whereas raising plasma carnosine levels seems very difficult in humans due to the high and human-specific serum carnosinase activity (Everaert 2012).

In the current study, we will investigate whether carnosine and/or beta-alanine supplementation can prevent high-fat diet-induced inflammatory signaling in skeletal muscle (local effect) and markers of (glyc)oxidation in plasma (systemic effect), and whether this prevents disturbed glucose transport in skeletal muscle and whole body glucose tolerance. In order to capture the early signs of possible protective effects of carnosine, our hypothesis will be tested in a very mild and initial stage of the metabolic disease development (high-fat diet induced rat model), in contrast to previous studies on severe diabetic models (Lee 2005; Sauerhofer 2007; Soliman 2007; Aldini 2011).

MATERIALS AND METHODS

Animals and diet

45% fat study: Three-week-old male Wistar rats (n= 24) were obtained from KU Leuven animal breeding center. Rats were housed per two in cages at 22°C in a 12 h light/dark cycle and were given ad libitum access to standard chow and water. After 1 week of acclimation, the rats were randomly assigned to three experimental groups receiving a specific diet for 14 weeks. Group 1 (CON) served as a control group and received a normal diet (Harlan Tecklad diet; 06416; 10% kJ fat, 20% kJ protein, 70% kJ carbohydrates), while the other two groups received a high-fat (HF) diet (Harlan Tecklad diet; 06415; 45% kJ fat, 19% kJ protein, 36% kJ carbohydrates). The second group (HF) had access to the high-fat diet and tap water and the third group (HFcar) additionally received 1.8% carnosine (Flamma) in their drinking water (80 mM). This concentration (1.8%) was chosen, as Everaert et al. (2013) has shown that this concentration results in a significant increase in carnosine (+ 57%) and anserine (+30%) in m. extensor digitorum longus in mice.

60% fat study: Three-week-old male Sprague-Dawley rats (n=36) were housed as described above. The rats were randomly assigned to four experimental groups receiving a specific diet for 8 successive weeks. Group 1 (CON) served as a control group and received a normal diet (Charles River: D01060501: 10% kJ fat), while the other three groups received a high-fat (HF) diet with 60% of energy in the form of fat (Charles River: D12492). Group 2 (HF) had access to pure water, group 3 (HFcar) additionally received 1.8% carnosine in their drinking water (Flamma, 80 mM) and the last group (HFba) 1% beta-alanine in their drinking water (Sigma, 112mM). A dose of 1% beta-alanine was chosen as Everaert et al. (2013) has shown that 1.2% beta-alanine results in a sign increase in carnosine (+156%) and anserine (+46%) in m. extensor digitorum longus in mice. Both diets (CON and HF diet) were similar concerning the protein (20% kJ, lactic casein), vitamin and mineral content, but differed for carbohydrates (CON: 70% kJ carbohydrates: 55gr corn starch and 12gr maltodextrin/100gr diet; HF: 20% kJ carbohydrates: 16gr maltodextrin and 9gr sucrose/100gr diet) and fats (CON: 1,9 gr lard and 2,4 gr soybean oil; HF: 31.5 gr lard and 3.2 gr soybean oil/100gr diet). Up to now, no data exist about a possible effect of carbohydrates on muscle carnosine baseline concentration.

This study (45% and 60% fat study) was approved by the animal Ethics Committee of KU Leuven and the housing conditions were as specified by the Belgian Law of November 14, 1993 on the protection of laboratory animals (agreement no. LA 1220548).

Experimental design

The aim of the initial 45% fat study was to 1) examine whether muscle carnosine increases after carnosine supplementation (80 mM of the drinking water) and 2) induce overweighed rats with concomitantly metabolic problems. We found only minimal effects on body weight with 45% HF feeding, therefore we did a follow-up study using a more dramatic high-fat diet (60% fat instead of 45% fat) and a fourth group (HFba) was added to test the role of plasma versus muscle carnosine as beta-alanine supplementation can increase muscle carnosine without an increase in plasma carnosine. In the 45% fat study, we collected 24h-urine in metabolic cages. However, since this negatively impacted the food intake and weight gain, we did not repeat the urine collection in the 60% study.

45% fat study: During the 14-week dietary intervention body weight, food and water (with or without BA) intake were recorded. At the end of the intervention, rats were individually housed in metabolic cages with their specific diet for 24 hours in order to collect a 24h urine sample. Thereafter, rats were terminally anaesthetized by an overdose of pentobarbital sodium (Nembutal; Brussels; Belgium) through an intra-peritoneal injection and two muscle types (white gastrocnemius and soleus) were dissected.

60% fat study: During the 8-week dietary intervention body weight, food and water (pure water, with BA or CAR) intake were recorded. At the end of the intervention, rats were surgically prepared for an intravenous glucose tolerance test (IVGTT) and blood was taken during the surgery. After an overnight fast, the IVGTT was performed. The rats were then allowed to recover from the IVGTT and received their specific experimental diet for 5 more days. Following an overnight fast, hindlimb perfusions were performed to assess glucose transport and three muscle types (red gastrocnemius, white gastrocnemius and soleus) were dissected.

Intravenous glucose tolerance tests

Rats were anaesthetized and surgically prepared for the IVGTT, as described by Vaisy et al. (2011).After an overnight fast (16-18 h) conscious rats were injected with 0.8 g glucose.kg^{-1} body weight using a 30%($w.v^{-1}$) glucose solution in ($w.v^{-1}$) saline. Blood glucose and plasma insulin concentrations were measured at regular time points until 120 min after glucose injection (according to Vaisy et al. (2011)). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from the paired ratio between plasma glucose and plasma insulin (Aldini 2011). Total area under the curve (AUC) for glucose, insulin and HOMA-IR was calculated using the trapezoidal rule.

Rat hindlimb perfusions and glucose transport

Following an overnight fast, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Hindlimb perfusion was performed as described by Wojtaszewski et al. (38) and slightly adjusted by Brandt et al. (2010), and was performed in three phases. Following surgical preparation, the hindlimbs were allowed to recover for 12 min (phase 1) with a cell-free perfusate recirculation at a flow of 20 ml/min (medium A). During phase 2, the right hindlimb is tied off and muscles are dissected. The left hindlimb was allowed to recover for 10 min with a one-way flow of 12,5ml/min with medium B, which was identical to medium A (basal glucose transport) or with 1000 µU/ml insulin (insulin stimulated glucose transport). Thereafter (phase 3), the glucose-free recovery perfusate was exchanged for medium C, an isotopic glucose-rich perfusate, for 12 min. At the end, the muscles of the left hindlimbs were dissected, frozen in liquid nitrogen and stored at -80°C. This sample was used for the determination of muscle glucose transport as previously described in detail (Wojtaszewski 1996). Radioactivity was measured in a liquid scintillation counter (1600 TR Liquid Scintillator Analyzer; Packard).

Muscle sampling

The muscle sampling included the white gastrocnemius (GW, a medial superficial part of the gastrocnemius consisiting mainly of fast-glycolytic fibers), the red gastrocnemius (GR, a deep proximal and medial portion of m. gastrocnemius, consisting mainly of fast-

oxidative fibers) and the m. soleus (SOL, consisting mainly of slow-oxidative fibers) (Delp 1996) from the right and left hindlimb.

Analyses on muscle

Quantification of histidine-containing dipeptides by means of HPLC (high-performance liquid chromatography). Skeletal muscles were freeze-dried (dw) and dissolved in phosphate buffer (1mg dw muscle/100µL PBS) for homogenization. Muscle homogenates were deproteinized using 35% sulfosalicylic acid (SSA) and centrifuged (5min, 14000g). 100µL of deproteinized supernatant was dried under vacuum (40°C). Dried residues were resolved with 40µL of coupling reagent:

Methanol/triethylamine/H_2O/phenylisothiocyanate (PITC) (7/1/1/1) and allowed to react for 20 minutes at room temperature. The samples were dried again and resolved in 100µL of sodium acetate buffer (10mM, pH 6.4). The same method was applied to the combined standard solutions of carnosine (Flamma) and anserine (Sigma). The derivatized samples (20 µL) were chromatographed on a Waters HPLC system with a Spherisorb C18/ODS2 column (4.6 x 150 mm, 5 µm), ODS2 guard column (80Å, 5 µm, 4.6 mm X 10 mm) and UV detector (wavelength: 210 nm). The columns were equilibrated with buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid) and buffer B (60% acetonitrile-40% buffer A) at a flow rate of 0.8 ml/min at 25°C. The limit of quantification was 7 µM.

RT-qPCR for quantification of TNFα and iNOS mRNA levels. Total RNA from rat soleus muscle was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). Briefly, skeletal muscle tissue was homogenized in Trizol Reagent using a tissue homogenizer. Total RNA was isolated from these homogenates according to the manufacturer's instructions. Concentration and purity of RNA samples was determined using a ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). Reverse transcription was performed on 0.5 µg of total mRNA using the Takara Prime Script cDNA Synthesis kit (Takara Bio Europe, Saint-Germain-en-Laye, France) according to the manufacturer's instructions.

For real time cDNA amplification we used SYBR Green Mastermix (Roche Applied Science, Penzberg, Germany) and the following primers: rat TNF-α: acaaggctgccccgactatgtgctc (fw), gatggcggagaggaggctgactttc (rev); rat iNOS: cccagagtctctagacctcaaca (fw), catggtgaacacgttcttgg (rev); rat HPRT: ctcatggactgattatggacaggac (fw),

gcaggtcagcaaagaacttatagcc (rev) ; rat RPL19: caatgccaactctcgtcaacag (fw), catccaggtcaccttctcgg (rev).

The samples were analyzed using the Light Cycler 480 (Roche Applied Science, Penzberg, Germany). To control the specificity of amplification, data melting curves were inspected. Normalization of gene expression values and selection of reference genes (GeNorm) was performed using the QbasePLUS software package (Biogazelle, Ghent, Belgium). According to GeNorm analysis, normalization to two reference genes (HPRT and RPL19) was optimal for our dataset.

Blood and urine biochemistry

Whole blood glucose and plasma insulin concentrations were determined using respectively a glucose analyzer (glucocard X-meter) and a sensitive rat insulin RIA kit (Linco Research). The enzymatic-colorimetric kits from Sentinel Diagnostic (Milan, Italy) were used for the analysis of *total cholesterol* (#17644) and *albumin* (#17600).

Advanced oxidation protein products (AOPP) were quantified according to the method previously described by Witko-Sarsat et al. (1998). Briefly, plasma samples were diluted in PBS and 200 µl aliquots pipped into each well of a 96-well microtitre plate and acidified by adding 20 µl of acetic acid to each well. Standards were prepared by adding 10 µl of 1.16 M potassium iodide (Sigma, Milan, Italy) to 200 µl of chloramine-T solution (0 to 100 µmol/l) (Sigma, Milan, Italy) and then spiked with 20 µl of acetic acid. The absorbance of the reaction mixture was read after 2 minutes at 340 nm on a Wallac Victor ^{2} Wallac 1420 workstation microplate reader. AOPP concentrations are expressed as µM of chloramine-T equivalents.

AGEs and ALEs analyses. The plasma and urine content of Nε-(carboxymethyl) lysine (CML) protein adduct was measured by the use of a commercially available ELISA kit (OxiSelect™ N^{ε}-(carboxymethyl) lysine (CML)ELISA Kit, Cell Biolabs Inc). Plasma and urine were diluted and tested in duplicate. Measurement of 4-hydroxy-2-nonenal (HNE) protein adducts in plasma was performed using OxiSelect™ HNE Adduct ELISA Kit, (Cell Biolabs Inc. Valter Occhiena S.R.L., Torino, Italy). The ELISA assayes were performed according to the manufacturer's instructions and the absorbance read on a Wallac Victor ^{2} Wallac 1420 workstation microplate reader. Plasma and urinary fulorescent AGEs were determined by

using a spectrofluorimeter (Perkin Elmer LS50B) setting the excitation wavelenth at 370 nm and emission at 440 nm. AGE values were expressed as fluorescence units (FU)/ml for plasma samples and in 24 hour for urines.

Carnosine and Carnosine HNE adduct (CAR-HNE) by HPLC-ESI-MS/MS analysis. Plasma and urinary content of carnosine and of the corresponding HNE-Michael adduct (CAR-HNE) was determined by LC-ESI-MS/MS in multiple reaction monitoring mode (MRM) mode as already reported (Orioli 2007). Briefly, to each sample, 10 µL of a H-Tyr-His-OH solution (500 µM solution in 1 mM PBS, pH 7.4) was added as an internal standard and the samples were then deproteinized by adding 110 µL of 700 mM perchloric acid solution. After 15 min at 4 °C, the samples were centrifuged (26 000g, 10 min), and the supernatants were diluted (1:1 v/v) with the mobile phase A (water:acetonitrile:heptafluorobutyric acid ; 90:10:0.1, v/v/v), filtered and analysed by LC-MS/MS. LC-ESI-MS analyses were carried out by using a TSQ Quantum triple-quadrupole mass spectrometer (ThermoFinnigan Italia, Milan, Italy) with electrospray ionization (ESI) source connected to a HPLC ThermoFinnigan Surveyor LC system equipped with a quaternary pump, a Surveyor autosampler, a vacuum degasser. Mass spectrometric analyses were performed in positive ion mode. Chromatographic separations were done by reverse phase elution with a Phenomenex Sinergy polar-RP column (150 mm 9 2 mm i.d.; particle size 4 lm) (Chemtek Analytica, Anzola Emilia, Italy) protected by a polar-RP guard column (4 mm 9 2 mm i.d.; 4 lm) kept at 25° C. Separations were done by gradient elution from 100% phase A to 80% phase B (CH_3CN) in 12 min at a flow rate of 0.2 ml min^{-1} (injection volume 10 µl); the composition of the eluent was then restored to 100% A within 1 min, and the system was re-equilibrated for 6 min. The samples rack was maintained at 4 °C. ESI interface parameters were set as follows: middle position; capillary temperature 270 °C; spray voltage 4.0 kV. Nitrogen was used as nebulizing gas at the following pressure: sheath gas 30 psi; auxiliary gas 5 a.u. Mass spectrometric analyses were performed in positive ion mode and in MRM mode using the following parent ion -> product ions transitions were used for quantitative analysis:

carnosine: 227. 1 m/z -> 110.1 m/z + 156.6 m/z (collision energy, 38 eV);

CAR-HNE: 383.1 m/z -> 110.1 m/z + 266.2 m/z (collision energy 40 eV);

H-Tyr-His-OH : 319.2 m/z -> 156.6 m/z + 301.1 m/z (collision energy, 25 eV).

Statistics

A general linear model repeated measures ANOVA was used to evaluate body weight over time. The effects of the different supplementation protocols on delta body weight, the weight of the epididymal fat pads, average energy intake, drinking behavior, urine and blood parameters, carnosine and anserine concentration in skeletal muscle, glucose transport and mRNA expression in skeletal muscle were analyzed with a one-way analysis of variance (ANOVA). When a significant group effect was shown, post hoc LSD tests were performed. All analyses were done with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p < 0.05$.

RESULTS

Body and fat mass

45% fat study. Although epididymal fat pads were significantly bigger in HF (HF: 18.7 ± 6.4g; HFcar: 17.0 ± 5.5g) compared to CON (13.1 ± 3.8g; p< 0.05), we were not able to report significant effects of the high-fat diet on body weight (CON: 430 ± 34g; HF: 480 ± 69g; HFcar: 445 ± 71g; p = 0.254). Carnosine supplementation did not influence these parameters.

In the 60% fat study, body weight and the epididymal fat pads of the HF groups were increased compared to the CON group by respectively +12% and +77% (table 1, p < 0.05), only the weight of the epididymal fat pads of the HF group was not significantly increased. Carnosine (HFcar) and beta-alanine (HFba) supplementation did not significantly change the effect of the high-fat diet on body and fat mass.

	CON	HF	HFcar	HFba
Start Body Weight (g)	153 (± 16)	155 (±8)	157 (± 10)	155 (± 11)
End Body Weight (g)	486 (± 57)	545 (± 47)*	572 (± 47)*	584 (± 60)*
Delta Body Weight (g)	334 (± 48)	390 (± 44)*	415 (± 44)*	429 (± 53)*
Weight Epididymal Fat pads (g)	8.9 (± 2.9)	13.8 (± 6.2)	16.9 (± 6.3)*	16.5 (± 5.9)*
Average Energy Intake (kJ/day/cage)	761 (± 158)	882 (± 87)$	942 (± 50)*	996 (± 102)*

Table 1. *60% fat study*: Body weight, epididymal fat and average energy intake over 8 weeks for CON, HF and HF treated groups (HFcar, HFba). Values are expressed as mean ± SD. * p<0.05 vs CON, $ p<0.1 vs CON.

Dietary intake

45% fat study. All rats had a similar energy intake (CON: 620 ± 59, HF: 643 ± 69, HFcar: 613 ± 95 kJ/day/cage), although it appeared that HFcar drank more (~15%) than the non-supplemented groups (HF and CON), but this was only significant for weeks 5, 11 and 14. Taking into account their average body weight for a period of 14 weeks, the rats from the HFcar group ingested 1468 (± 196) mg carnosine/kg BW per day.

60%fat study. The HF groups (HF, HFcar and HFba) had a significantly higher energy intake (+24%) compared to the CON group (p < 0.05, table 1). Both HFcar and HFba had a similar drinking behavior and averaged ~37 ml/day during the 8-week intervention, which

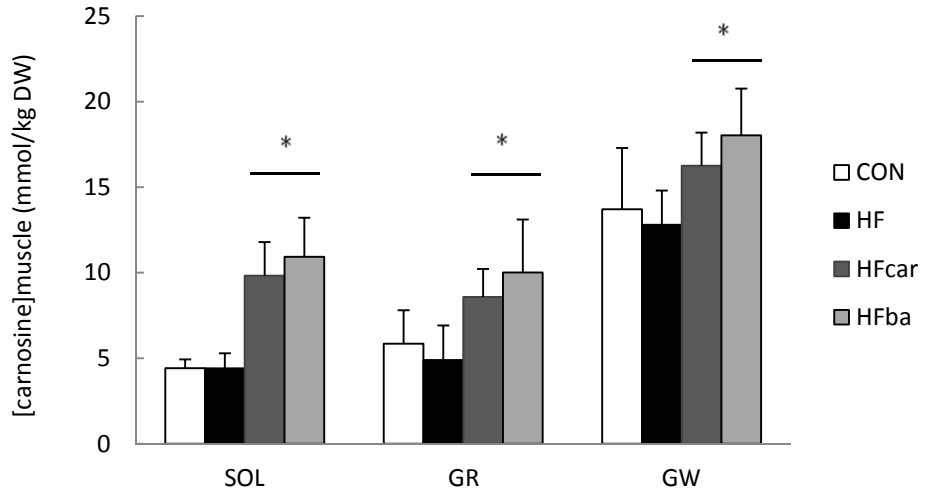
resulted in a carnosine intake of 1697 (± 175) mg /kg BW per day and a beta-alanine intake of 933 (± 58) mg /kg BW per day, respectively.

Histidine-containing dipeptides in plasma, urine and muscles.

45% fat study. In the carnosine-supplemented group (HFcar) urinary carnosine concentrations reached levels of 342.1 ± 419.7 nmol/ml, whereas no carnosine was detected in urine in the non-supplemented groups (CON and HF) (table 2). Carnosine supplementation increased muscle carnosine (SOL: +4.8 mmol/kg DW, GW: +6.5 mmol/kg DW) and muscle anserine in SOL (+2.8 mmol/kg DW), but not in GW (-0.4 mmol/kg DW) compared to HF (data not shown). HF diet alone did not decrease muscle carnosine nor anserine.

60% fat study. The plasma carnosine concentration of the CON, HF and HFba ranged from below limit of quantification (BLQ) (n=3/24) to 1.72 nmol/ml (table 3). However, when carnosine was supplemented (HFcar), plasma concentrations reached levels of 15.1 ± 18.5 nmol/ml. As in the 45% fat study, HF diet alone did not change muscle carnosine nor anserine levels. Muscle carnosine content was increased by both carnosine (HFcar) and beta-alanine (HFba) supplementation, with no significant differences between those two supplemented groups and no significant difference between the three muscle types (HFcar: SOL: +5.5, GR: +3.7, GW: +3.5 ; HFba: SOL: +6.6, GR: +5.2, GW: +5.2, all expressed as mmol/kg DW) (figure 1). As SOL and GR have lower baseline values, the relative increase is markedly higher for muscles with a high percentage of type I fibers (SOL: +123% and GR: +75%) compared to muscles with a high percentage of type II fibers (GW: +34%) (figure 1A). A similar pattern was observed for muscle anserine, the methylated form of carnosine (figure 1B). The average absolute increase of muscle anserine in HFcar and HFba compared to HF was ~10.0 mmol/kg DW, with relative increases of +87% for SOL, +58% for GR and +22% for GW (figure 1B). The sum of muscle carnosine and muscle anserine is designated as histidine-containing dipeptides (HCD). The absolute increase of HCD was similar for the three muscle types, but tended to be higher in the HFba group compared to HFcar, with respectively +18.6 mmol/kg DW and +11.3 mmol/kg DW (p=0.07).

1A.



1B.

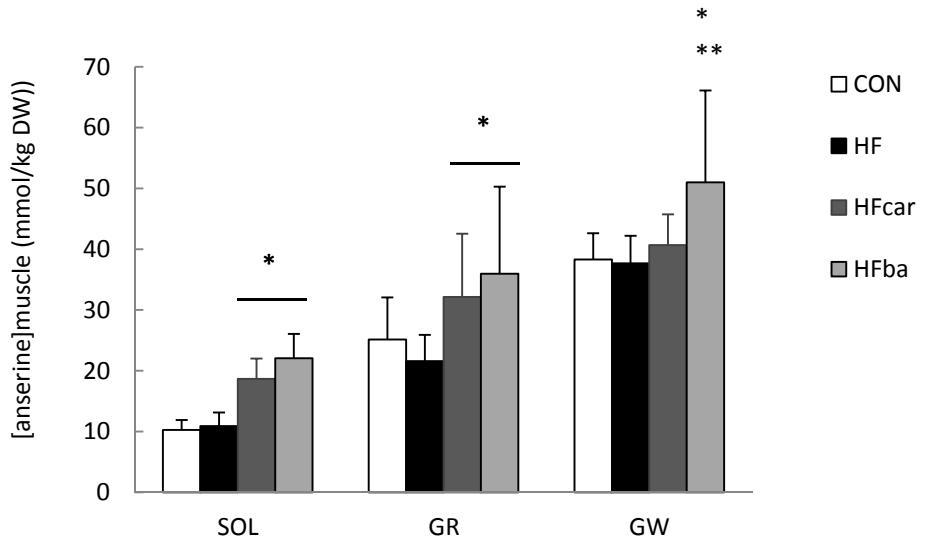


Figure 1. *60% fat study*: Muscle carnosine (1A) and muscle anserine (1B) in the m. soleus (SOL)**,** m. gastrocnemius red (GR) and in m. gastrocnemius white (GW). * $p<0.05$ vs HF and ** $p<0.05$ vs HFcar

Inflammatory and glycoxidative markers in plasma, urine and skeletal muscle.

45% fat study. Metabolic urine profile of the rats is given in table 2. HF diet induced marked elevation of urinary carboxymethyl-lysine (CML) concentration ($p < 0.05$), which was prevented by almost 50% through carnosine administration. No differences were present for total AGEs. Albumin excretion in the urine increased, although not significantly, in HF and reduced to a great extend in HFcar compared to HF. Carnosine-HNE, a measure for the quenching ability of carnosine towards HNE, was detected in urine for 3 out of 8 rats in HFcar only.

URINE PROFILE PER 24 H	CON	HF	HFcar
Urine volume (ml/24h)	23.7 (± 7.9)	22.6 (± 4.2)	21.0 (± 10.9)
CML (µg/24h)	9.8 (± 4.5)	35.5 (± 15.0)**	18.4 (± 15.9)*
Total AGEs (U.F./24h)	99723 (± 31135)	82799 (± 23919)	92583 (± 43668)
Albumin (mg/24h)	296 (± 320)	535 (± 461)	129 (± 196)*
Carnosine (nmol/24h)	/	/	7729 (± 14901)
Carnosine-HNE (nmol/24h)	/	/	0.145 (± 0.084)

Table 2. *45% fat study:* 24-h urine profile of CON, HF and HFcar groups. Rats had access to their specific diet. Values are expressed as mean ± SD. * $p<0.05$ vs HF, ** $p<0.05$ vs CON and HFcar.

In plasma (60% fat study), a similar pattern was found (table 3). Carnosine supplementation decreased plasma CML with more than 50% compared to the HF group (HF: 1.80 ± 0.95 nmol/ml, HFcar: 0.78 ± 0.92 nmol/ml, $p < 0.05$), whereas beta-alanine supplementation had no effect (1.65 ± 1.29 nmol/ml). Carnosine-HNE was detected in plasma for 2 out of 9 rats in HFcar only. The other metabolic markers (total cholesterol, AOPP, HNE protein adducts and total AGEs) were not significantly changed by HF diet, nor by supplementation (HFcar and HFba). In figure 2, two mediators of chronic inflammation (TNFα and iNOS) in skeletal muscle are shown. Carnosine, but not beta-alanine, supplementation decreased the mRNA expression of iNOS in m. soleus by 46.9% compared to HF ($p < 0.05$) and tended to decrease soleus TNFα mRNA by 25.7% compared to HFba ($p= 0.072$).

PLASMA PROFILE	CON	HF	HFcar	HFba
CML (nmoles/ml)	1.06 (± 1.12)	1.80 (± 0.95)	0.78 (± 0.92) *	1.65 (± 1.29)
Total AGEs (U.F./ml)	500.2 (± 162.8)	424.0 (± 79.7)	363.1 (± 80.8)	363.7 (± 75.7)
Total Cholesterol (mg/dl)	52.2 (± 9.3)	62.5 (± 10.9) $	57.4 (± 11.6)	60.8 (± 15.3)
AOPP (µM eq.Cl.T)	290.2 (± 168.0)	279.3 (± 58.9)	314.6 (± 110.5)	312.4 (± 175.0)
HNE (nmoles/ml)	1.59 (± 1.20)	1.09 (± 1.10)	1.36 (± 1.12)	0.91 (± 1.16)
Carnosine (nmol/ml)	0.6 (± 0.4)	0.6 (± 0.5)	15.1 (± 18.5) **	0.5 (± 0.4)
Carnosine -HNE (nmol/ml)	/	/	0.010 ± 0.000 (2/9)	/

Table 3. *60% fat study:* Metabolic markers, plasma carnosine and carnosine adducts in plasma of the rats (fasted for 4 hours). Values are expressed as mean ± SD. ** p<0.05 vs CON, HF and HFba, * p<0.05 vs HF and $ p<0.1 vs CON.

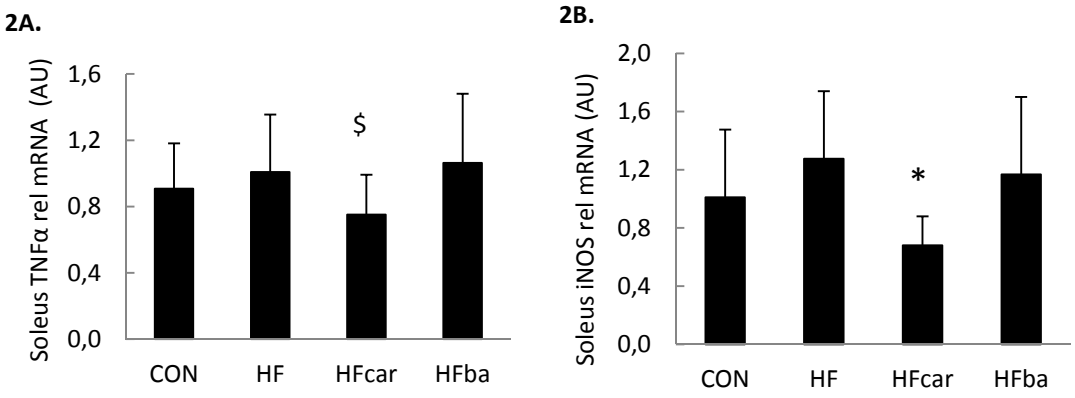


Figure 2. *60% fat study:* Relative mRNA expression of TNFα and iNOS in m. soleus in CON, HF and the two intervention groups (HFcar and HFba). Values are expressed as mean ± SD. * p<0.05 vs HF and HFba, $ p<0.1 vs HFba.

In vivo whole body glucose tolerance (IVGTT) and skeletal muscle glucose transport

60% fat study. Fasting plasma glucose and the area under the curve (AUC) of plasma glucose during the IVGTT were not significantly altered through HF diet, nor by supplementation (HFcar and HFba) (figure 3A and 3C). On the other hand, fasting plasma insulin was decreased by 45% by carnosine supplementation, but not by BA supplementation compared to HF ($p < 0.05$, figure 3B). The AUC of plasma insulin was more than doubled in the HFba group compared to the CON group ($p < 0.05$, figure 3D).

The AUC of HOMA-IR was higher in the three HF groups (HF, HFcar and HFba) compared to the CON group ($p < 0.05$, figure 3E).

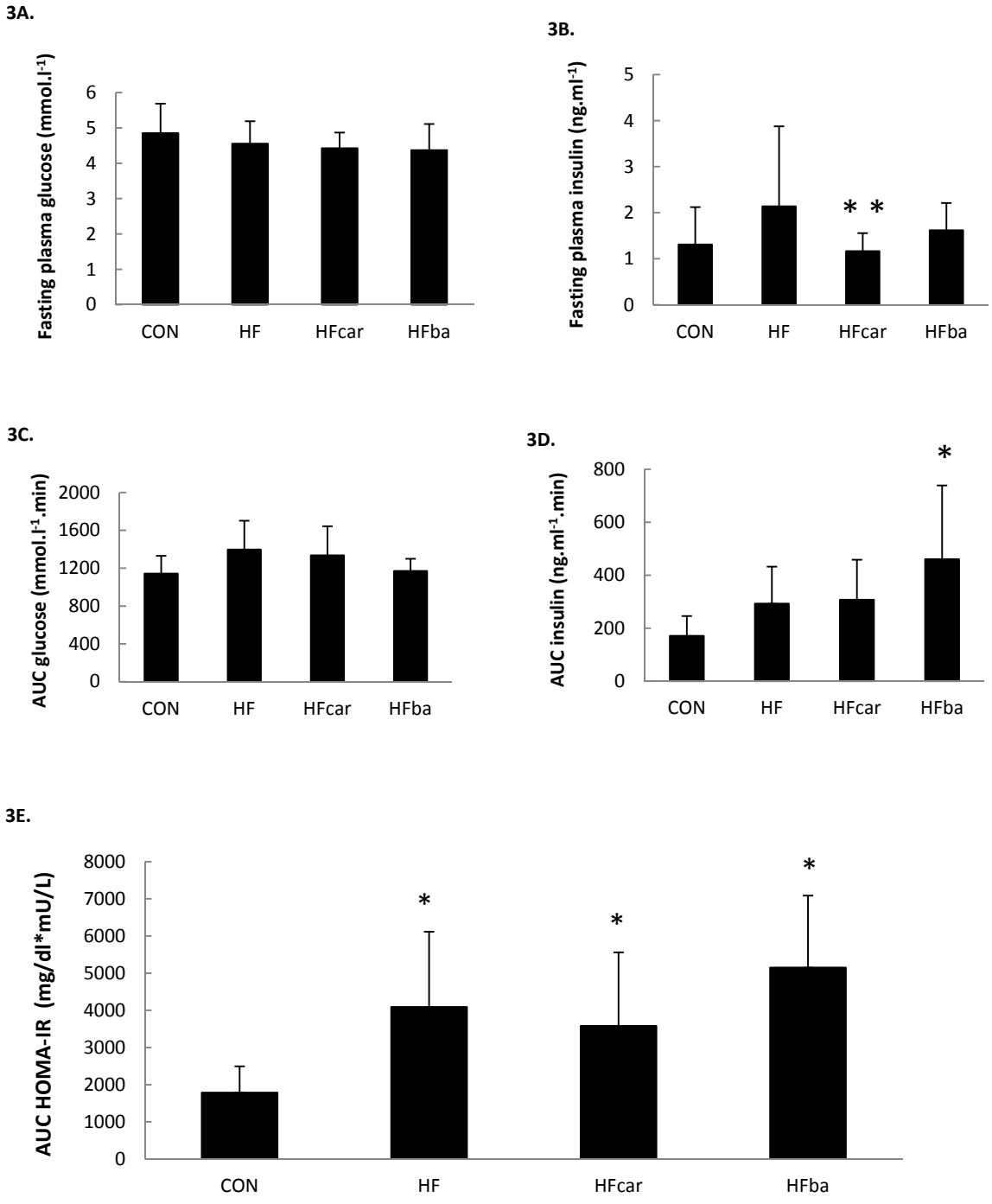


Figure 3. *60% fat study:* Intravenous glucose tolerance test (IVGTT). Fig 3A and 3B represent fasting plasma glucose and insulin, respectively. Fig 3C, 3D and 3E represent the area under the curve (AUC) for plasma glucose, insulin and HOMA-IR during the IVGTT. Values are expressed as mean ± SD. * $p<0.05$ vs CON and ** $p<0.05$ vs HF.

Insulin stimulated glucose transport in skeletal muscle was increased by 3- to 4-fold compared to basal glucose transport in every experimental group (CON, HF, HFcar and HFba) ($p < 0.05$), however, no effects of high-fat diet and supplementation (HFcar and HFba) were detected (table 4).

	Basal glucose transport (µmol/g/h)	**Insulin stimulated glucose transport (µmol/g/h)**	**Delta (µmol/g/h)**
CON	2.0 (± 0.4)	7.9 (± 1.9) *	5.9 (± 1.9)
HF	3.0 (± 0.7)	7.8 (± 3.7) *	4.9 (± 3.7)
HFcar	3.2 (± 0.1)	7.6 (± 4.5) *	4.4 (± 4.5)
HFba	1.5 (± 0.9)	8.9 (± 2.9) *	7.4 (± 2.9)

Table 4. *60% fat study:* Basal (± 3 rats per group) and insulin (± 6 rats per group) stimulated glucose transport in m. plantaris. Values are expressed as mean ± SD. *p<0.05 vs basal glucose transport.

DISCUSSION

The first pathophysiological signs of HF feeding and the prevention by carnosine

Potential therapeutic effects of carnosine have so far only been investigated on severe and advanced models of diabetes and diabetic complications (Yamano 2001; Nagai 2003; Lee 2005; Sauerhofer 2007). Given the multitude of biochemical properties of carnosine (Boldyrev 2013), possibly involved in the protective effects, and the multitude of physiological and biochemical abnormalities in these severe diabetic models, it is difficult to determine what is directly and what is indirectly affected by carnosine administration. We have aimed to look at the effects of carnosine in the initial stage of metabolic disturbance. In the 45% HF model, despite not yet attaining significant overweight, the first signs of the protective effects of carnosine were already observed, mainly by reducing urinary CML by 50% and by decreasing microalbuminuria with 75% compared to HF, but also by reporting CARN-HNE adducts in urine of some HFcar rats. Similar findings (concerning reduction of CML and CARN-HNE adducts) are found in the plasma of our overweight 60% HF model. A very recent and prominent paper of Baba et al. (2013) confirmed the presence of CAR-HNE adducts in mice and humans and reported the discovery of a metabolic pathway leading to the removal of these conjugates. The enzyme aldose reductase (AR) catalyzes the reduction of carnosine-aldehyde conjugates (carnosine-propanals) into carnosine-propanols in lysates of heart, skeletal muscle and brain tissue from wild-type, but not AR-null mice. Concerning the suppressive effect of carnosine supplementation on microalbuminuria (induced by chronic HFdiet), it does not only point towards a protective effect on kidney damage, but the retention of albumin in the circulation could increase whole body anti-oxidant capacity as albumin plays an important role in scavenging free radicals (Caraceni 1994).

In our study, total AGEs were not (yet) altered by HF feeding, which can be explained by the fact that CML can derive from glyoxal, which is a by-product of either glycoxidation or lipid-oxidation (Vistoli 2013). On the other hand, total AGEs determined by fluorescence mainly come from glycoxidation. So, it seems that there are no oxidation products from glycoxidation in the plasma of these rats, but more from lipidoxidation and this is in agreement with the fat-rich animal diet and with the fact that the animals were

euglycemic. Taken together, these data on oxidative damage well indicate that the model we used is characterized by a very initial stage of oxidative stress since the AOPP levels did not change significantly as well as the protein HNE adducts as determined by ELISA. Only CAR-HNE metabolites determined by using the high specific and selective LC-ESI-MS methods were determined in some animals. Unlike Aldini et al. (2011), who saw marked effects of carnosine on dyslipidemia in Zucker obese rats, we could not confirm it at the level of plasma total cholesterol, although this could be explained by the fact that total cholesterol was also not (yet) markedly altered by our 60% HF diet (p=0.08). Interestingly, metabolic improvements induced by carnosine were not secondary to the prevention of overeating/obesity, which was a confounding factor in the study of Aldini et al. (2011). We can therefore conclude that carnosine has direct metabolic effects.

Muscle Carnosine or Plasma Carnosine approach?

A second aim of the current study was to identify whether beneficial effects are secondary to elevated plasma or elevated tissue carnosine concentrations. Our study design and results allowed to investigate this, as the HFcar had both elevated plasma and tissue (muscle) carnosine content, whereas HFba had only elevated muscle carnosine, without elevated plasma carnosine content. Here, we demonstrated for the first time that plasma carnosine (HFcar), and not muscle carnosine (HFba), is protective against HF diet-induced lipidoxidation in plasma and urine (systemic effects) and for inflammatory signaling in muscle (target organ). Sustained increased inflammatory signalling in skeletal muscle has been shown to induce muscle insulin resistance (Pillon 2012). The fact that only HFcar and not HFba protected skeletal muscle from overweight-induced inflammatory signalling (although both groups equally increased muscle carnosine above normal levels), clearly points to a systemic effect with skeletal muscle as a target organ. This would suggest that skeletal muscle cannot be protected from metabolic stress by further raising its carnosine content. Muscle carnosine levels were not reduced by HF feeding in the present study, suggesting that there is no need to ‘restore’ normal levels, which is in contrast with previous reports in the literature (Buse 1980; Gualano 2012).

One possible and likely mechanism by which systemic changes (like plasma CML) can influence inflammatory signaling in muscle/tissues is through the AGE-RAGE axis. CML is

an important ligand for RAGE and this AGE-RAGE interaction triggers intracellular events increasing nuclear transcription factors such as nuclear factor-kappa B (NF-κB), resulting in increased transcription of e.g. TNFα (Yan 1994; Kislinger 1999; Haslbeck 2005) and concomitantly increased iNOS expression (Ramasamy 2005). The finding that carnosine has a systemic effect, rather than a local effect, creates an obstacle in the road to developing a carnosine-related treatment for diabetic patients, as the highly active enzyme serum carnosinase (CN1) almost immediately hydrolyses carnosine in plasma into its two constituent amino acids beta-alanine and L-histidine upon oral administration (Everaert 2012). Further research must, however, first point out whether beta-alanine supplementation induces carnosine synthesis in other organs, such as retina and kidney, and whether or not this protects the respective organs.

Glycemic Control

Up to now, studies on the effect of carnosine on glycemic control have been mainly performed in diabetes type 1 models, such as streptozotocin-treated rats (Yamano 2001; Nagai 2003; Soliman 2007) and Balb/cA mice (Lee 2005). All studies reported that carnosine supplementation increased plasma insulin in the fasted state or during a glucose tolerance test, concomitant with decreased levels of glucose. The study of Sauerhofer et al. (2007) is the only one using a type 2 diabetic model (db/db mice) and they report similar effects as in type 1 models. In this light, our study is novel as it demonstrates another mechanism in a HF diet rat model. Fasting plasma glucose was not (yet) affected by high-fat diet, nor by HFcar or HFba, but fasting plasma insulin was reduced by ~45% in the carnosine supplemented HF group compared to HF, suggesting a better basal whole body insulin sensitivity. This study did not induce clear effects on glucose tolerance during the IVGTT and on glucose transport in skeletal muscle, therefore we cannot assess the effect of carnosine and beta-alanine supplementation. Additionally, it is noteworthy that chronic beta-alanine supplementation induced a markedly enhanced insulin response during the IVGTT. Possibly, this could be related to the fact that some amino acids can acutely and chronically regulate insulin secretion from pancreatic β-cells (Newsholme 2006), but the specific role of beta-amino acids, such as beta-alanine, has not yet been investigated.

Conclusions

We designed for the first time a study that allows us to distinguish between the effects of elevated plasma and tissue carnosine or elevated tissue carnosine alone, by supplementing respectively carnosine or beta-alanine. From this study we can conclude that plasma carnosine and not muscle (tissue) carnosine is involved in preventing lipid-oxidation in plasma and inflammatory signaling in skeletal muscle upon HF feeding.

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Study 4

GLUCOSE INTOLERANCE IS ASSOCIATED WITH INCREASED MUSCLE HISTIDINE-CONTAINING DIPEPTIDES IN BOTH RODENTS AND HUMANS

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Manuscript in progress

ABSTRACT

Introduction. Muscle carnosine and its methylated form anserine are histidine-containing dipeptides (HCDs). Both dipeptides have the ability to quench reactive carbonyl species and it is suggested that tissue levels are decreased in pathological circumstances, such as diabetes. Yet, some reports found conflicting findings in this regard.

Methods. Rodent study: Skeletal muscles of rats and mice were collected from 4 different diet-intervention studies, aiming to induce various degrees of glucose intolerance: 45% high-fat (HF) feeding (rats), 60%HF feeding (rats), cafeteria feeding (rats) and 70%HF feeding (mice). The 70%HF intervention enclosed a third group, HF70%+ex, exercising 5x1h/week for 6 weeks. Body weight (BW), glucose-tolerance and muscle HCDs were assessed. Human study: Muscles biopsies are taken from the Vastus Lateralis in 35 non-vegetarian males (9 lean, 8 obese, 9 prediabetic patients (preT2D), 9 diabetic type 2 (T2D) patients) and muscle carnosine was measured.

Results. Rodent study: Muscle HCDs changed (increased) only in the more drastic diet-interventions inducing clear increases in body weight and glucose-intolerance (cafeteria and 70%HF feeding). Human study: Muscle carnosine of the obese, preT2D and T2D increased with 21% ($p>0.1$), 30% ($p<0.05$) and 39% ($p<0.05$), respectively, compared to the lean.

Conclusion. Muscle HCDs increase with progressive glucose intolerance, both in rodents (interventional) and humans (cross-sectional). This implicates that the quenching ability of HCDs is of minor importance in determining its content in skeletal muscle. Therefore, this paper provides several diabetes-related factors possibly influencing muscle carnosine content in a diabetic state.

Keywords: quenching ability, advanced lipoxidation and glycation endproducts,

carnosinylated proteins.

INTRODUCTION

Histidine-containing dipeptides (HCDs) are peptides consisting of a histidine (or a methylated form of histidine) and the atypical amino acid beta-alanine (Boldyrev 1990). HCDs are predominantly and abundantly present in skeletal muscle (Zapp 1938; Boldyrev 1990; Boldyrev 2012), but they are also measurable in other tissues, although in concentrations 10- to 1000-fold lower (Boldyrev 2006; Kamal 2009; Peters 2011; Boldyrev 2013). Human skeletal muscles only possess the HCD carnosine (beta-alanyl-L-histidine), whereas rodents have carnosine along with its methylated variant anserine (beta-alanyl-N-π-methylhistidine) (Davey CL 1960; Crush 1970; Tamaki 1977).

Carnosine supplementation has recently been associated with a later and milder evolution of diabetes in type 1 and 2 diabetic rodent models (Sauerhofer 2007; Soliman 2007; Pfister 2011; Riedl 2011). The mechanism has been attributed to carnosine's physiological characteristic to increase insulin secretion (Yamano 2001; Nagai 2003; Sauerhofer 2007), but also to its biochemical property to quench and scavenge damaging species such as reactive oxygen species (Decker 2000), metal-ions (Dobbie H 1955; Mineo 2002), protein carbonyls (Hipkiss 2002) and reactive carbonyl species (RCS) by forming adducts with 4-hydroxy-2-nonenal (HNE) (Carini 2003) and acrolein (Orioli 2005). Until recently, these properties of carnosine were only confirmed in vitro. Aldini et al. (2011) was the first to report that Zucker Obese rats have higher levels of carnosine-HNE adducts in urine compared to their lean counterparts, which points to HNE overproduction in the obese animals and confirms the role of carnosine as an endogenous detoxifying agent of RCS such as HNE. In addition, Baba et al. (2013) confirmed the presence of carnosine-RCS conjugates (HNE and acrolein) in urine from normal, healthy, non-smoking adults and demonstrated its existence in skeletal muscle of C57BL/6 mice. Interestingly, anserine has similar characteristics as carnosine (Kohen 1988; Egorov 1997; Fontana 2002; Orioli 2005; Szwergold 2005), although less intensively investigated.

As consequence of these scavenging characteristics, it is suggested that carnosine levels in diabetic tissues are decreased, as it has been shown that diabetic tissues have high production of reactive oxygen and carbonyl species (Brownlee 2001; Brownlee 2005; Samjoo 2013). Indeed, decreased carnosine and anserine levels are demonstrated in

retina, kidney and liver of obese and diabetic (type 1 and 2) rodents (Pfister 2011; Riedl 2011; Peters 2011; Mong 2011). However, for skeletal muscle, contradiction exists. In rodents, there is only one paper available which shows decreased carnosine levels in diaphragm muscle of STZ-induced diabetic rats (Buse 1980). In humans, Gualano et al. (2012) reported reduced gastrocnemius carnosine content (-45%) in 14 type 2 diabetic patients (but not in soleus muscle nor in type 1 diabetic patients) compared to 14 control subjects matched for age, BMI, gender and diet. However, no consensus is reached, as another study (Srikanthan 2012) demonstrated an increase in soleus carnosine in 10 diabetic type 2 patients (patients on oral and insulin therapy were excluded) compared to non-diabetic controls.

Although evidence is scarce, a new research topic about elevating tissue/muscle carnosine in clinical populations is emerging because of its physiological and scavenging properties. Not only by supplementing carnosine in clinical circumstances, but also by proposing beta-alanine as a good alternative (Gualano 2012; Del Favero S 2012; Sale 2013), because it is the rate-limiting precursor for carnosine synthesis in muscle (Harris 2006). Therefore, this study is designed to elucidate whether skeletal muscle HCDs are affected by diet-induced obesity and glucose intolerance. Moreover, as it is known that exercise can reverse metabolic stress in skeletal muscle (Kiens 2006; Pedersen 2012), the additional effect of daily endurance training will be studied.

METHODS

Study Design

Rodent study (intervention study). Skeletal muscles of rats and mice were collected from 4 different diet-intervention studies, aiming to induce various degrees of glucose intolerance. The specifications of the animals and the diets are presented in table 1.

					Energy content of the diet (% total kJ)		
Study	Species	Strain	Gender	Intervention period	Macro-nutrients	CON	HF/CAF
Rat studies							
HF 45%	Rats	Wistar	M	14 weeks	Total CHO	70	36
					Total fat	10	45 (mainly lard)
					Total protein	20	19
HF 60%	Rats	Sprague-Dawley	M	8 weeks	Total CHO	70	20
					Total fat	10	60 (mainly lard)
					Total protein	20	20
CAF	Rats	Wistar	M	12 weeks	Total CHO	63	69 (mainly sugar)
					Total fat	12	16
					Total protein	25	15
Mouse study							
HF 70%	Mice	C57BL/6J	F	6 weeks	Total CHO	standard chow	<1
					Total fat		72 (lard + corn oil)
					Total protein		28

Table 1: *Rodent study:* Specifications of the animals and diets for the rat and mouse studies. Abbreviations: M: male, F: female, CHO: carbohydrates

Shortly, in the HF 45% study, 15 male Wistar rats received either a 45% high-fat diet (HF 45%, n=8) or a control diet (CON, n=7) during 14 weeks. The high fat diet contained 45% fat of total kJ, mainly coming from lard (Harlan Tecklad diets, CON:06416, HF:06415). In the HF 60% study, 18 male Sprague-dawley rats received either a 60% high-fat diet (HF 60%, n=9) or a control diet (CON, n=9). Here as well, the fat from the diet was mainly coming from lard, but the % of total kJ increased up to 60% (Charles River diets, CON: D01060501, HF: D12492). In the CAF study, 22 male Wistar rats received either a cafeteria diet (CAF, n=11) or a control diet (CON, n=11) during 12 weeks. The cafeteria diet is another type of the obesity-inducing diets, because of its high amount of simple sugars (48% of total kJ) and was prepared from powdered rodent pellets (Muracon-G, Carfil Quality, Oud-Turnhout, Belgium; 330 g/kg), full fat sweetened condensed milk (Nestlé, Vevey, Switzerland; 330 g/kg), sucrose (70 g/kg), and water (270 g/kg) (Vaisy 2011). In the HF 70% study, 22 female C57BL/6J mice received either a control diet (CON, n=8), a 70%

high-fat diet (HF 70%, n=8) or a HF 70% diet in combination with endurance training (12 m/min for 60 min per day, 5 days a week) (HF 70%+ex, n=6), during 6 weeks. Seventy-two % of total kJ was fat and mainly derived from lard (Deldicque 2013). All rodents were housed at 22°C in a 12 h light/dark cycle and were given ad libitum access to their water and specific diet.

In all rodent studies, body weight was recorded during the intervention study and glucose intolerance was measured at the end of the intervention (cfr infra for the description of the glucose tolerance tests). Thereafter, all animals were sacrificed by an intra-peritoneal injection of sodium pentobarbital solution and muscles were dissected for the determination of muscle HCDs (carnosine and anserine). In the rat studies, the muscle sampling included the white gastrocnemius (GW, a medial superficial part of the gastrocnemius consisting mainly of fast-glycolytic fibers) and the red gastrocnemius (GR, a deep proximal and medial portion of m. gastrocnemius, consisting mainly of fast-oxidative fibers) (Delp 1996). In the mouse study, the gastrocnemius was taken as a whole (GAS), because it is difficult to make a visual distinction between the GR and GW in mice. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until HCD content analysis. All rodent studies were approved by the local animal Ethical Committee (the rat studies by KU Leuven, the mouse study by Université catholique de Louvain).

Human study (cross-sectional study). Muscle biopsies were taken from the Vastus Lateralis in 35 non-vegetarian males with different degrees in body weight and glucose intolerance: 9 lean, 8 obese, 9 prediabetic patients (preT2D), 9 diabetic type 2 patients (T2D: diet treated only). Glucose tolerance was classified as defined by the American Diabetes Association 2006 (blood glucose at 2 hours OGTT for lean and obese: < 140 mg/dl; preT2D: > 140mg/dl and < 200mg/dl; T2D: > 200mg/dl). Subjects were age-matched across the groups, with a mean age of 45 ± 7 yrs. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until carnosine content analysis. Subjects gave their informed consent and the study was approved by the local Ethical Committee.

Glucose tolerance tests

Rodent study. The intravenous glucose tolerance tests (IVGTT) in the *rat studies* (the HF 45%, HF 60% and CAF-study) were performed according to Vaisy et al. (2011). Briefly, rats were anaesthetized and surgically prepared for the IVGTT, which involved catheter insertion into the left jugular vein. After an overnight fast (16-18 h) a glucose solution (1g glucose.kg-1 body weight using a 30% w.v.-1 glucose solution in w.v-1 saline) was injected into the catheter of the conscious rats and blood glucose and plasma insulin concentrations were measured at regular intervals. In the HF 45% and the HF 60% study, blood glucose was determined at time points 0, 5, 10, 15, 30,60, 90, and 120 min after glucose injection and plasma insulin at 0, 10, 30, and 90 min after glucose injection. In the CAF study, blood was taken at 0, 10, 30 and 90 min for glucose and insulin determination. Total $AUC_{glucose}$ and $AUC_{insulin}$ were calculated using the trapezoidal rule.

The oral glucose tolerance tests (OGTT) from the *mouse study* is described in Deldicque et al. (2013). Plasma glucose was determined on time points: 30 min before and 0, 15, 30, 60, 90 and 120 min following oral glucose gavage, plasma insulin was only determined 30 min before and 15min after glucose gavage. Total AUC for glucose ($AUC_{glucose}$) was calculated using the trapezoidal rule.

Quantification of muscle carnosine, anserine and total HCDs

HCDs (carnosine and anserine) were quantified by means of reversed-phase HPLC (high-performance liquid chromatography). Skeletal muscles were dissolved in phosphate buffer (rat studies: 1mg dw muscle/100µL PBS; mouse study: 10mg ww muscle/100 µLPBS; human study: 1mg ww muscle/15 µL PBS) for homogenization. Muscle homogenates were deproteinized using 35% sulfosalicylic acid (SSA) and centrifuged (5min, 14000g). 100µL of deproteinized supernatant was dried under vacuum (40°C). Dried residues were resolved with 40µL of coupling reagent: methanol/triethylamine/H2O/phenylisothiocyanate (PITC) (7/1/1/1) and allowed to react for 20 minutes at room temperature. The samples were dried again and resolved in 100µL of sodium acetate buffer (10mM, pH 6.4). The same method was applied to the combined standard solutions of carnosine (Flamma) and anserine (Sigma). The derivatized samples (20 µL) were chromatographed on a Waters HPLC system with ODS2 guard column (80Å,

5 µm, 4.6 mm X 10 mm), a Spherisorb C18/ODS2 column (4.6 x 150 mm, 5 µm), and UV detector (wavelength: 210 nm). The columns were equilibrated with buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid) and buffer B (60% acetonitrile-40% buffer A) at a flow rate of 0.8 ml/min at 25°C. The limit of quantification of muscle homogenates was 7 µM (~0,7 mmol/kg DW muscle). Total HCDs are the sum of muscle carnosine and anserine.

Statistics

Rodent study. To compare body weight, $AUC_{glucose}$, $AUC_{insulin}$, muscle carnosine, anserine and HCDs between the HF/CAF groups (HF 45%, HF 60%, CAF or HF 70%) and their own control group, data were analyzed by an independent t-test. Z-scores were calculated for every rat/mouse of the high-fat/cafeteria group to compare muscle carnosine levels of these obese rats between the 4 intervention studies. Z-scores were calculated using mean and standard deviation of the specific control groups. To compare the z-scores of muscle carnosine, anserine and HCDs between the four HF/CAF groups, This statistical analysis was also used to compare muscle carnosine, anserine and HCD between the 3 groups (CON, HF 70% and HF 70%+ex) in the HF 70% study.

Human study. To compare muscle carnosine between the 4 groups (lean, obese, preT2D and T2D), a one-way ANOVA was performed. When a significant group effect was shown, a post hoc LSD test was done. All analyses (human and rodent) were done with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p < 0.05$.

RESULTS

Body weight and glucose tolerance (table 2)

Rodent study: In the *HF 45% study*, end body weight tended (p=0.08) to be higher in HF compared to CON, but glucose tolerance ($AUC_{glucose}$ and $AUC_{insulin}$) was not significantly altered. In the *60% HF study*, end body weight and $AUC_{insulin}$ was higher in HF (+ 12% and + 71% respectively, p<0.05) compared to CON, and $AUC_{glucose}$ tended to be higher (+ 22%, p=0.054). In the *CAF study,* body weight, $AUC_{glucose}$ and $AUC_{insulin}$ were all higher in HF compared to CON (+ 12%, 25% and 139% respectively, p<0.05). In the *HF 70% study*, both body weight and $AUC_{glucose}$ were higher in HF compared to CON (+ 14% and + 81% respectively, p<0.05). In high-fat diet mice subjected to an endurance exercise program (HF 70%+ex), body weight was normalized back to levels of CON mice, however, $AUC_{glucose}$ could not be decreased by exercise. Plasma insulin at time points -30 min and +15 min from the OGTT was not different between the three groups.

Study		Obesity		Glucose tolerance test			
		Delta body weight (g)	End body weight (g)	Total AUC for glucose		Total AUC for insulin	
				mmol.l^{-1}.min	% increase	ng.ml^{-1}.min	% increase
Rat studies							
HF 45%	CON (n=8)	323 ± 32	430 ± 34	999 ± 138		463 ± 134	
	HF (n=7)	377 ± 61 *	480 ± 69 $	1038 ± 127	4	533 ± 246	15
HF 60%	CON (n=9)	334 ± 48	486 ± 57	1144 ± 186		171 ± 74	
	HF (n=9)	390 ± 44 *	545 ± 47 *	1397 ± 305 $	22	293 ± 139 *	71
CAF[a]	CON (n=11)	255 ± 14	490 ± 47	579 ± 29		185 ± 28	
	CAF (n=11)	305 ± 10*	548 ± 55 *	721 ± 44 *	25	443 ± 39 *	139
Mouse study							
HF 70%[a]	CON (n=8)	3.9 ± 1.0	21.6 ± 1.9	168 ± 68			
	HF (n=8)	6.1 ± 1.1 *	23.1 ± 1.5	304 ± 127 *	81	not available	
	HF + ex (n=6)	4.7 ± 1.3 **	20.7 ± 1.4**	280 ± 71 *	67		

Table 2: Rodent study: Degree of obesity and glucose tolerance in the 4 interventions. Values are expressed as mean ± SD. * p<0.05 versus CON, ** p<0.05 HF+ex versus HF and $ p<0.1 versus CON. Underlined % increase (compared to CON) are significant with p<0.05. aData from the CAF and HF 70% study are obtained from respectively Vaisy et al. (2011) and Deldicque et al. (2013).

Human study. Body weight and BMI of the lean subjects was significantly lower compared to the other three groups (body weight: lean: 78±10, obese: 99±21, preT2D: 103±8, T2D: 97±12 kg; BMI: lean: 25±1, obese: 29±3, preT2D: 32±2, T2D: 31±3 kg/m^2).

Muscle carnosine, anserine and HCDs (table 3)

Rodent study: In the *HF 45%* and *HF 60% study*, neither muscle carnosine, nor anserine, nor the sum (total HCDs) was altered by high-fat feeding (for both muscle types GR and GW). In the *CAF study*, muscle anserine and total HCDs tended to increase in GW by 14% (p=0.085) and 12% (p=0.076), respectively. No changes were reported for muscle carnosine and GR. In the *HF 70% study*, muscle carnosine significantly increased with 30% (p<0.05) and muscle HCDs tended to increase (+21%) in HF compared to CON (p= 0.095). Muscle anserine followed a similar pattern, but no significant results were obtained. Daily exercise training (HF 70%+ex) seemed to counteract the high-fat diet-induced increase in muscle anserine and total HCDs (p=0.082) compared to HF.

		Carnosine mmol/kg DW		**Anserine** mmol/kg DW		**HCD** mmol/kg DW	
Study	Group	**GW**	**GR**	**GW**	**GR**	**GW**	**GR**
Rat studies							
HF 45%	CON (n=8)	19.1 ± 4.1	9.2 ± 2.1	34.6 ± 2.4	29.2 ± 7.6	53.7 ± 3.6	38.5 ± 7.8
	HF (n=7)	18.2 ± 4.0	8.3 ± 1.4	35.7 ± 6.5	29.4 ± 5.4	53.9 ± 9.3	37.7 ± 6.0
HF 60%	CON (n=9)	13.7 ± 3.6	5.9 ± 2.0	38.3 ± 4.3	25.4 ± 7.0	52.0 ± 4.9	31.3 ± 7.3
	HF (n=9)	12.8 ± 2.0	4.9 ± 2.0	37.7 ± 4.5	21.8 ± 4.4	50.5 ± 4.7	26.7 ± 4.1
CAF	CON (n=11)	9.7 ± 1.8	7.1 ± 0.9	30.4 ± 5.1	24.0 ± 4.0	40.1 ± 6.4	31.1 ± 4.9
	CAF (n=11)	10.5 ± 2.3	7.9 ± 1.3	34.6 ± 5.7 $	23.3 ± 3.1	45.1 ± 6.2 $	31.2 ± 3.9
Mouse study							
		GAS		**GAS**		**GAS**	
HF 70%	CON (n=8)	5.4 ± 1.3		11.1 ± 2.4		16.4 ± 3.6	
	HF (n=8)	7.0 ± 1.7 *		12.9 ± 2.5		19.9 ± 4.1 $	
	HF+ex (n=6)	5.7 ± 1.1		10.7 ± 1.7 $$		16.4 ± 2.6 $$	

Table 3: Rodent study: Muscle carnosine, anserine and total HCD for the animals of the rat (HF 45%, HF 60% and CAF) and mouse studies (HF 70%). Values are expressed as mean ± SD. * p< 0.05 HF versus CON, $ p< 0.1 HF vs CON and $$ p<0.1 HF+ex vs HF. GW, GR and GAS represent the white, the red and the gastrocnemius as a whole, respectively.

If the z-scores for muscle HCDs are compared between the 4 intervention diets, we observe higher z-scores in the groups with marked glucose intolerance (CAF and HF 70%) compared to the groups with no or limited glucose intolerance (HF 45% and HF 60%) (figure 1). This pattern is similar for muscle carnosine (HF 45%: -0.42 ± 0.97, HF 60%: -0.32 ± 0.80, CAF: 0.87 ± 1.25, HF 70%: 1.24 ± 1.30) and muscle anserine (HF 45%: 0.14 ± 0.53, HF 60%: -0.49 ± 0.87, CAF: 0.52 ± 0.62, HF 70%: 0.78 ± 1.06) separately.

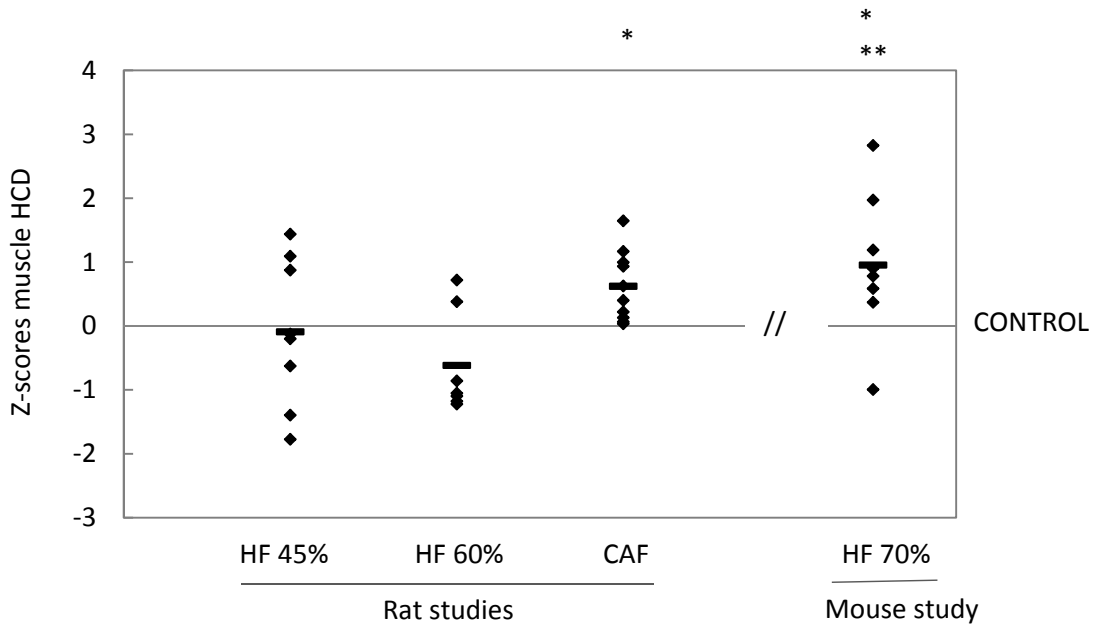

Figure 1: *Rodent study*: Z-scores of total muscle HCDs for the groups HF 45%, HF 60%, CAF and HF 70% compared to their own control group (zero-line). Diamonds and stripes represent individual and group values, respectively. * p< 0.05 vs HF 60%, ** p< 0.05 vs HF 45%.

Human study. Muscle carnosine gradually increased with increasing glucose intolerance. Muscle carnosine of the obese, preT2D and T2D is higher [+21% (p>0.1), +30% (p<0.05) and +39% (p<0.05), respectively], compared to the lean (figure 2).

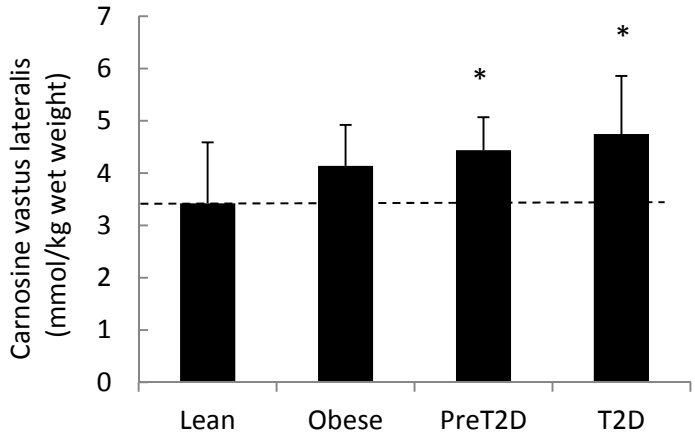

Figure 2: *Human study*: Muscle carnosine content in lean, obese, prediabetic patients (preT2D) and diabetic type 2 patients (T2D). Subjects are age-matched across the groups. Values are expressed as mean ± SD. * p<0.05 versus control.

DISCUSSION

According to the 'scavenging theory', it is currently believed that carnosine levels in diabetic tissues are decreased. Briefly, carnosine sacrifices itself to scavenge reactive carbonyls species such as HNE and acrolein in vivo (Aldini 2011; Baba 2013), species which are abundantly present in diabetic tissues (Samjoo 2013). Indeed, a decrease in carnosine content has been reported in organs such as retina, kidney and liver (Pfister 2011; Riedl 2011; Mong 2011), however, literature about carnosine levels in diabetic muscles are contradictive. In collaboration with a Brazilian research group, we previously reported a decrease in muscle carnosine (Gualano 2012), whereas others found an increase (Srikanthan 2012). Therefore, we have examined the effect of different degrees of glucose intolerance on muscle carnosine and anserine in both rodents (diet-intervention study) and humans (cross-sectional study with lean, obese, preT2D patients and T2D patients). This paper shows that muscle HCDs increase with progressive glucose intolerance.

In the search for explaining this increase, we first looked at the known determinants of muscle carnosine and whether or not glucose intolerance could play an interfering role. Muscle fiber type is one of the most important determinants of muscle carnosine content (next to age and sex)(Parkhouse 1985; Harris 1998; Everaert 2011; Baguet 2012). Muscles with a high portion of fast glycolytic type II fibers possess more (almost a double) carnosine and anserine compared to muscles with a higher portion of oxidative type I fibers (Harris 1998; Hill 2007; Baguet 2011; Everaert 2013b). Obesity and diabetes have several times been associated with alterations in muscle fiber type distribution, yet with opposing findings. On the one hand, insulin resistance and diabetes are associated with an elevated portion of fast glycolytic type II muscle fibers (Oberbach 2006; Stuart 2013). On the other hand, several studies have demonstrated that treatment of lean animals with a high-fat diet is followed by muscle fiber alterations in favor of oxidative metabolism including increased proportions of type I fibers in skeletal muscle. This, in an attempt to remove the overload of fat delivery (Abou 1992; Shortreed 2009). These apparently opposing findings may be the sequential outcome of a complex mechanism that regulates excess of fat availability, being different for the initial phase compared to the long-term phase. In line with this, Yasuda et al. (2002) demonstrated a higher % of

type I fibers (and a lower % of type IIA fibers) in soleus muscle of 5- and 9 week-old Goto-Kakizaki rats (non-obese diabetic rats). On the other hand, an increased % of type IIB fibers was observed in plantaris muscle of 9- and 20-week-old Goto-Kakizaki rats (Yasuda 2002). Interestingly, this study (HF 70% study) could demonstrate that an endurance exercise program (in glucose-intolerant mice possessing elevated muscle HCDs) tended to normalize muscle HCDs. As endurance exercise training is known to stimulate or preserve slow type I fibers, it is likely that muscle fiber type is accounted for this normalization.

Our paper does not exclude the 'self-sacrificial theory' in other tissues and probably it is also present in skeletal muscle, we only suggest that it is of minor importance in skeletal muscle. Carnosine concentrations are, as already mentioned, a 1000-fold higher in skeletal muscle compared to other tissues such as kidney and liver, and the highly active enzyme carnosine synthase, which is more expressed in the fast glycolytic type II fibers, will probably easily restore HCD levels if necessary. Moreover, it is possible that a compensation mechanism is activated by initially decreased muscle carnosine levels, ultimately leading to net increased carnosine levels.

However, the 'self-sacrificial theory' itself can be questioned as no evidence is provided for a possible degradation pathway of the subsequently formed carnosine-adducts. As a result, net carnosine levels would remain equal. But why did Gualano et al. (2012) found a decrease in muscle carnosine in Brazilian diabetes type 2 patients? Two possible factors can be put forward. First, low testosterone levels are common in men with obesity and type 2 diabetes and may be associated with insulin resistance (Grossmann 2008; Grossmann 2013). In addition, muscle carnosine levels have been shown to be negatively modified by orchidectomy in male mice (Penafiel 2004; Everaert 2013a). Second, we have recently shown that meal and beta-alanine co-ingestion enhances muscle carnosine loading in healthy subjects, suggesting that insulin plays a role (Stegen 2013). Meal-induced elevations in serum insulin are capable of stimulating the sodium-dependent beta-alanine transporter TauT, secondary to its action of increasing sacrolemmal Na+/K+-ATPase pump activity (Clausen 2003), resulting in increased beta-alanine flux into the muscle. Extrapolating these data, it can be suggested that insulin resistance, a common hallmark of obesity and diabetes (Kahn 2003; DeFronzo 2009; Bergman 2012), has a

lowering effect on intramuscular beta-alanine availability and therefore muscle carnosine levels.

So, several factors explaining both an increase or decrease in muscle carnosine (or anserine) can be put forward to explain the existing and conflicting literature (cfr supra). However, this paper demonstrates both in rodents (interventional) and in humans (cross-sectional) that muscle HCDs increase with progressive glucose intolerance. These data implicate that caution is warranted to treat diabetic patients with beta-alanine as a deficit in muscle HCDs in this population is not assured. Moreover, we underscore the importance of future studies aiming to better understand the metabolic pathways and the (patho)physiological significance of HCD in muscle in particular and in the body in general.

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Study 5

SERUM CARNOSINASE CONTENT AND ACTIVITY

IN TYPE 2 DIABETIC PATIENTS.

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Manuscript in progress

ABSTRACT

Introduction. A polymorphism in the serum carnosinase gene (CNDP1), encoding for low serum carnosinase (CN1) activity, is associated with reduced risk of diabetic nephropathy. The substrates of CN1, carnosine and anserine, have been associated on their turn with a later and milder evolution of diabetes in type 1 and type 2 diabetic rodent models, when given as a supplement. These two findings have created the hypothesis that low CN1 activity levels are protective in the development of diabetes or diabetes-related diseases as it would increase the bioavailability of its substrates in the circulation. On the other hand, it is suggested that hyperglycemia increases CN1 activity, pointing towards the importance of good glycemic control in type 2 diabetic patients (T2D).

Aims. This study has two main aims: 1) to investigate whether T2D patients are indeed characterized by higher CN1 levels (content and activity) and whether exercise training or a better glycemic control could suppress CN1. 2) to explore the relationship between CN1 activity and lipid profile and blood pressure in T2D patients.

Methods. Plasma samples were taken from a large cohort *T2D patients* (153 men, 90 women, age:54.3±7.1 yrs) before and after a 6-month exercise training program (4 groups: remaining sedentary, aerobic exercise, resistance exercise and combined exercise training)(part of DARE clinical trial). Plasma CN1 content and activity, HbA1c, lipid profile and blood pressure were measured. In *healthy subjects* (90 men, 66 women, age: 24.4±7.5 yrs), CN1 content and activity was also determined.

Results and conclusions: We provided evidence that hyperglycemia does not elevate CN1 content and activity levels as 1) no correlation between CN1 and HbA1c is present in T2D patients and 2) there is no difference in CN1 content between healthy subjects and T2D patients, moreover CN1 activity was significantly higher in the healthy subjects. In addition, CN1 content and activity are stable over a 6-month period and not influenced by chronic exercise training. Finally, CN1 was associated with lipid profile, but findings were inconsistent between CN1 content and activity.

Keywords: CNDP1, carnosine, histidine-containing dipeptides, carnosinase specific activity

INTRODUCTION

Human serum carnosinase or CN1 belongs to the family of the M20 metalloproteinases and is secreted by the liver or brain into the circulation, where it is highly active in the adult human (Jackson 1991; Teufel 2003). It hydrolyses the histidine-containing dipeptides carnosine (β-alanyl-L-histidine), homocarnosine (γ-aminobutyryl-histidine) and anserine (β-alanyl-N-π-methylhistidine) back into its constituent amino acids, with the highest specificity for carnosine (Peters 2010; Peters 2011b). In the past decade, two different research approaches have associated this carnosine/carnosinase system with diabetes or diabetic complications.

First, carnosine supplementation has recently been associated with a later and milder evolution of diabetes in type 1 and 2 diabetic rodent models (Sauerhofer 2007; Soliman 2007; Pfister 2011; Riedl 2011; Peters 2011a). The mechanism has been attributed to carnosine's physiological and biochemical properties (Boldyrev 2013). Carnosine can interact with oxidative and glycative species (Hipkiss 1995; Decker 2000), but also with its deleterious derivatives in vivo, such as reactive carbonyl species (Aldini 2011; Baba 2013) and protein carbonyls (Baba 2013). As consequence, the formation of advanced glycation and lipoxidation end-products (AGEs and ALEs), which are abundantly present (and detrimental) in diabetes or diabetes-related diseases (Metz 2003), will be suppressed (Aldini 2011). From a physiological point of view, it has been demonstrated that carnosine increases insulin secretion in rodent diabetic models (Nagai 2003a; Sauerhofer 2007). Moreover, Lee et al. (2005) reported reduced triglyceride and cholesterol levels in heart and liver after carnosine supplementation in diabetic Balb/cA mice. Later animal studies could confirm beneficial effects of carnosine supplementation on dyslipidemia in both plasma and tissues (Mong 2011; Aldini 2011), together with an anti-atherogenic (Menini 2012) and anti-hypertensive effect (Aldini 2011).

Second, several genome-wide linkage scans for diabetic nephropathy and subsequently fine-mapping have demonstrated that some polymorphisms in the gene for CN1 (CNDP1) are closely associated with diabetic nephropathy susceptibility (Janssen 2005; Freedman 2007; Mooyaart 2010; Zhu 2013). More specifically, Caucasian diabetic patients homozygous for the 5 leucine allele were less susceptible to diabetic nephropathy

(especially women). In addition, homozygosity for the five-leucine allele of CNDP1 (the least CTG repeats) was associated with the lowest CN1 actvitiy and vice versa, the more CTG repeats in CNDP1 (the 6, 7 or 8 leucine allele), the higher the CN1 activity (Janssen 2005), but this was only demonstrated in healthy individuals. A later study on Cos-7 transfected cells (Riedl 2007) confirmed that the 5 leucine allele impairs the efficiency of secretion of serum carnosinase, resulting in lower CN1 activity.

Interestingly, both research approaches share the same working hypothesis, i.e. higher bioavailability of carnosine in the circulation, due to carnosine ingestion (by diet or supplementation) or lower CN1 activity, will provide more disease-protecting effects according to its biochemical properties. This brings us to our first hypothesis. We expect that diabetic patients having lower CN1 content and activity display a healthier lipid profile and blood pressure compared to patients having high CN1 content and activity.

Despite the relevance of this carnosine/carnosinase system in diabetes or diabetes related complications, little is known about the determinants of CN1 content and activity in type 2 diabetes. Therefore, this study will better characterize those determinants in this population. First, we expect that CN1 content and activity will be higher in women then in men, as demonstrated in healthy individuals (Bando 1984; Peters 2010; Everaert 2011). Second, we hypothesize that hemoglobin A1C (HbA1c) is positively correlated with CN1 content and activity. CN1 is a heavily glycosylated protein with three putative N-glycosylation sites (Teufel 2003), as consequence, Riedl et al. (2010) stepwise deleted of its putative N-glycosylation sites in Cos-7 cells and found that CN1 secretion and activity was decreased. In a next step Riedl et al. (2010) suggested that hyperglycemia would increase CN1 secretion/activity because of increased N-glycosylation. Hyperglycemia triggers the hexosamine pathway, leading to elevated UDP-N-acetylglucosamine (GlcNAc) concentrations (Brownlee 2001), which is a critical factor in the N-glycosylation (Sasai 2002). Indeed, they demonstrated that (CGT)5 homozygous diabetic (type 1 and 2) patients had increased CN1 activity levels compared to (CGT)5 homozygous healthy controls. In this light, it can be hypothesized that exercise training in diabetic patients, aiming for ameliorating insulin sensitivity and thereby lowering hyperglycemia and HbA1c, decreases CN1 content and activity (third assumption). Moreover, our lab has recently reported lower CN1 levels (both content and activity) in elite athletes, involved in high

intensity exercise, compared to untrained controls (Baguet 2014). However, the effect of exercise on CN1 has not yet been investigated.

For these purposes, we investigated CN1 content and activity in a large cohort diabetic type 2 patients (153 men and 90 women), before and after a 6-month intervention of exercise training (aerobic exercise, resistance exercise, combined aerobic and resistance exercise) or remaining sedentary (control). In addition, CN1 content and activity levels of diabetic type 2 patients will also be compared with healthy subjects (90 men and 66 women).

METHODS

Blood samples from patients with diabetes type 2 (153 men, 90 women) were taken from the DARE (Diabetes Aerobic and Resistance Exercise) clinical trial to measure CN1 content and activity before and after a 6-month exercise training program and compared with healthy subjects. The DARE clinical trial was originally designed to determine the effect of aerobic and resistance training alone (6 months) versus a sedentary control group, and the incremental effects of doing both types of exercise (combined exercise training) versus aerobic or resistance training alone, on glycemic control and other risk factors for cardiovascular disease (Sigal 2007). Design, participant characteristics and intervention are briefly described below and the reader is referred to the full manuscript of Sigal R.J. et al. (2007) for further details.

Design

Diabetes type 2 patients. A 26-week, single-center, randomized, control trial with parallel-group design was performed. Previously inactive persons with type 2 diabetes were randomly assigned to 1 of 4 groups: aerobic training, resistance training, combined aerobic and resistance training, or a control group that reverted to prestudy activity levels. Before and after the 6-month intervention period, heparin plasma blood samples were taken in fasted state and participants were instructed not to exercise 48 hours before each visit. The study was approved by the Ottawa Hospital Research Ethics Board, and all participants gave informed consent.

Healthy subjects. Heparin blood samples were collected to quantify plasma carnosinase protein content and activity (not in fasted state).

Participants

Diabetes type 2 patients. Baseline characteristics are presented in table 1. Inclusion criteria included type 2 diabetes (as defined by the American Diabetes Association (1997)) for more than 6 months and a baseline hemoglobin A1c value of 6.6% to 9.9% (normal range, 4.0% to 6.0%). Exclusion criteria were current insulin therapy; participation in exercise during the previous 6 months; changes during the previous 2 months in oral hypoglycemic, antihypertensive, or lipid-lowering agents or body weight (≥5%); serum creatinine level of 200 µmol/L or greater (≥2.26 mg/dL); proteinuria greater than 1 g/d;

blood pressure greater than 160/95 mm Hg; restrictions in physical activity because of disease; or presence of other medical conditions that made participation inadvisable. The exercise intervention took place at 8 community-based exercise facilities in the Ottawa–Gatineau region, Canada. Exercise was supervised by personal trainers.

Baseline Characteristics	Control group	Aerobic training group	Resistance training group	Combined exercise training group
Men/women, n/n	38/23	39/20	39/24	37/23
Mean age (SD), y	54,6 (7,1)	54,0 (6,6)	54,8 (7,6)	53,6 (7,2)
Mean hemoglobin A1c value (SD), %	7,66 (0,88)	7,69 (0,86)	7,72 (0,86)	7,66 (0,92)

Table 1. Baseline characteristics of diabetic type 2 patients

Healthy subjects. 156 healthy Belgian (Caucasian) subjects (90 men and 66 women) with a mean age of 24.4 yrs (SD ± 7.5) (age range: 18 – 69 yrs). All subjects were physically active (but not engaged in competitive sports or specifically trained) and nobody used medication. The control subjects were collected from two different studies from our lab (Everaert 2011, Baguet 2014).

Intervention

Diabetes type 2 patients. Exercise group participants exercised 3 times weekly, and training progressed gradually in duration and intensity. The aerobic training group exercised on treadmills or bicycle ergometers. Participants progressed from 15 to 20 minutes per session at 60% of the maximum heart rate to 45 minutes per session at 75% of the maximum heart rate, as determined by using a maximal treadmill exercise test. The resistance training group performed 7 different exercises on weight machines each session, progressing to 2 to 3 sets of each exercise at the maximum weight that could be lifted 7 to 9 times. The combined exercise training group did the full aerobic training program plus the full resistance training program to ensure an adequate dose of each type of exercise. Control participants were asked to revert to prestudy activity levels, had the same dietary intervention and spent the same time with the research coordinator and dietitian as did the participants from the exercise groups.

Plasma analyses

Glycemic control, lipid profile and blood pressure. Hemoglobin A1c was measured by using turbidimetric immunoinhibition, and total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were measured by using enzymatic methods on a Beckman-Coulter LX20 analyzer (Beckman Instruments, Brea, California). Low-density lipoprotein (LDL) cholesterol levels were calculated by using the Friedewald equation (Friedewald 1972). Blood pressure was measured after 10 minutes at rest; the mean of 2 readings obtained 2 minutes apart was used in statistical analysis.

CN1 content, activity and specific activity. CN1 concentrations (content) were determined by a sandwich ELISA (enzyme-linked immunosorbent assay). In brief, a human CN1 ELISA was developed by coating high absorbant microtitre plates (Greiner BioChemia, Flacht, Germany) overnight with 100 µl of goat polyclonal anti-human CN1 (1 µg/ml) (R&D, Wiesbaden Germany). The plates were extensively washed and incubated with 5 % w/v of dry milk powder to avoid unspecific binding. For each sample and standard, serial dilution was carried out. The plates were placed on a shaker for 1 hr and subsequently extensively washed with PBS/Tween. Hereafter, anti-human carnosinase monoclonal antibody (clone ATLAS, Abcam) was added for 1 hr followed by extensively washing. HRP conjugated goat anti-mouse IgG was added for 1 hr and the plates were washed. After addition of peroxidase substrate (deep-Blue POD) (Roche diagnostics, Mannheim, Germany) the reaction was stopped after 15 minutes by adding 50 µl of 1 M H_2SO_4 and read in an ELISA reader at 450 nm. CN1 protein concentrations were assessed in the linear part of the dilution curve. Sensitivity of the ELISA was approximately 20 ng/ml.

CN1 activity was determined according to a method described by Teufel et al. (2003). Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a heparin plasma sample and stopped after 10 min of incubation at 37 °C by adding 1 % SSA. Liberated histidine was derivatized with o-phtaldialdehyde (OPA) and the maximum increase was used for determining the maximum activity. Fluorescence was measured by excitation at 360 nm and emission at 460 nm.

CN1 specific activity is calculated by dividing CN1 activity by its content (the activity of an enzyme per milligram of total protein) and expressed as µmol/min/mg.

Statistical analysis.

At baseline, the pearson correlation coefficient was calculated to study 1) the relationship between CN1 content and CN1activity within subjects and 2) the effect of age on CN1 content and CN1 activity, separately for men and women. To investigate the intra-individual variation of CN1 content and activity over a 6-month period, the intraclass coefficient (single measures) was calculated for control subjects only. A two-sided independent-samples T-test was performed to compare CN1 content and activity between men and women at baseline. A linear regression analysis was performed to study the relationship between CN1 content/activity (independent variable) and glycemic control (HbA1c), the lipid profile (triglycerides, total cholesterol, HDL, LDL and the ratio total cholesterol/HDL) and blood pressure (SBP and DBP) (dependent variables). In this model, age and sex were taken as covariates and the natural logarithm of CN1content and activity was used because 1) the independent variables (CN1 content and activity) were skewly distributed and 2) the curve estimation between the independent and dependent variables showed a logarithmic function, rather than a linear function. The beta-coefficients are reported. Finally, the effect of a 6-month aerobic, resistance or combined exercise training on CN1 content and activity was investigated by a linear mixed model design, adjusted by gender, age and BMI. All analyses, except for the linear mixed model design, were done with SPSS statistical software (SPSS 20, Chicago, IL) and statistical significance was set at $p<0.05$.

RESULTS

CN1 in healthy versus T2D subjects

CN1 content is equal in healthy versus type 2 diabetic patients, with women having higher levels (~28%) than men in both populations (figure 1A). CN1 activity displays a different pattern between populations. In healthy subjects, women have 24% higher levels than men, but this difference disappears in type 2 diabetic patients. Moreover, CN1 activity levels are significantly lower in type 2 diabetic patients versus the healthy subjects, and more pronounced in the women (-35%) than in the men (-23%) (figure 1B). As consequence, CN1 specific activity is lower in type 2 diabetic patients compared to healthy subjects and more pronounced in diabetic women (-32%) than in diabetic men (-13%). Moreover, CN1 specific activity is equal between sexes in healthy subjects, but is lower in women than in men for the diabetic patients (figure 1C).

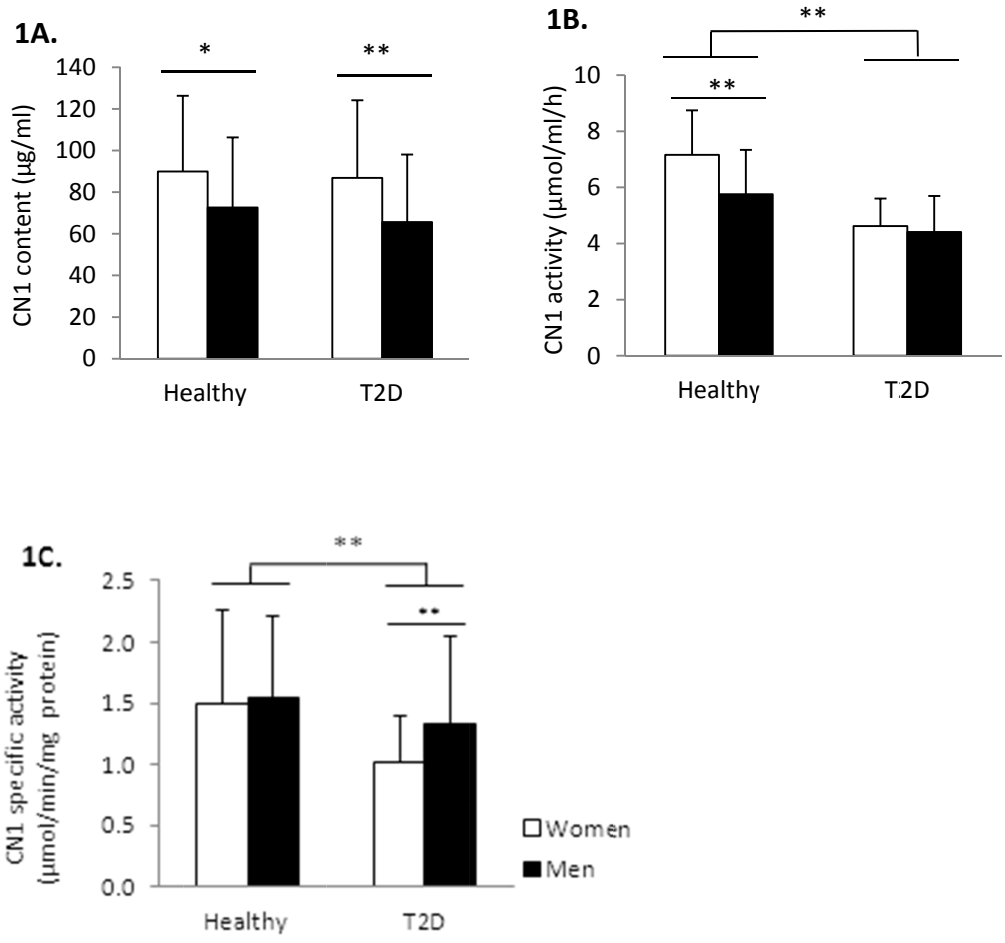
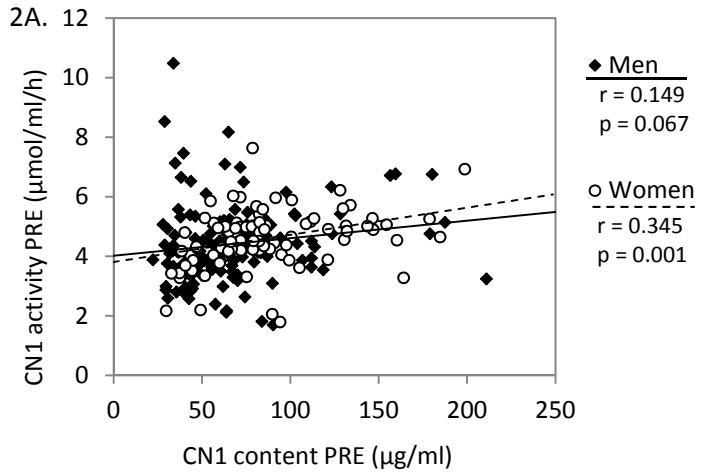
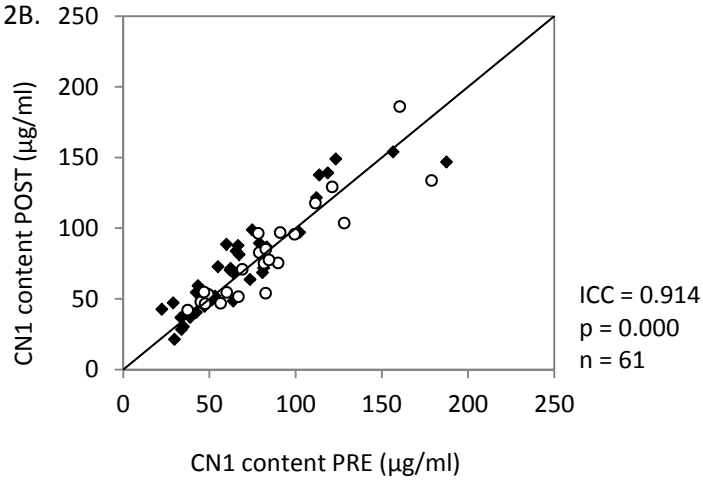


Figure 1. CN1 content (1A), CN1 activity (1B), CN1 specific activity (1C) for healthy and type 2 diabetic (T2D) men and women. * p-value < 0.01 and **p-value < 0.001.

Both populations demonstrate a low, but highly significant, correlation between CN1 content and activity at baseline (healthy subjects: r=0.464, p<0.001; T2D patients: r=0.222, p=0.001) (figure 2). When analyzing the data according to sex, this correlation is similar in healthy and diabetic women (healthy women: r=0.305, p=0.020; T2D women: r=0.345, p=0.001) and healthy men (r=0.469, p<0.001), but the correlation disappears in diabetic men (r=0.149, p=0.067).





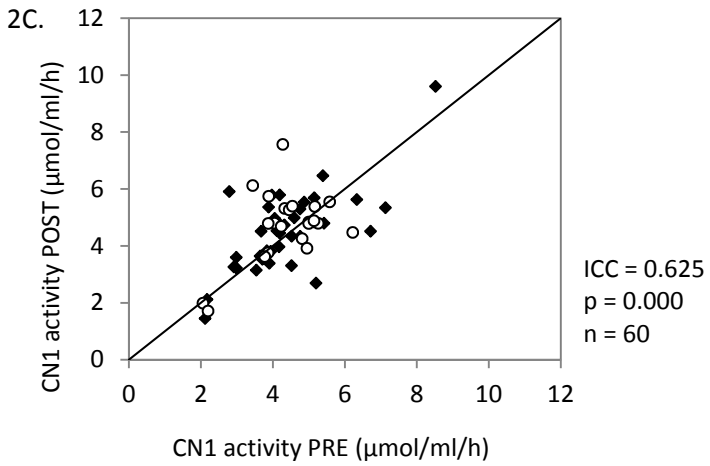
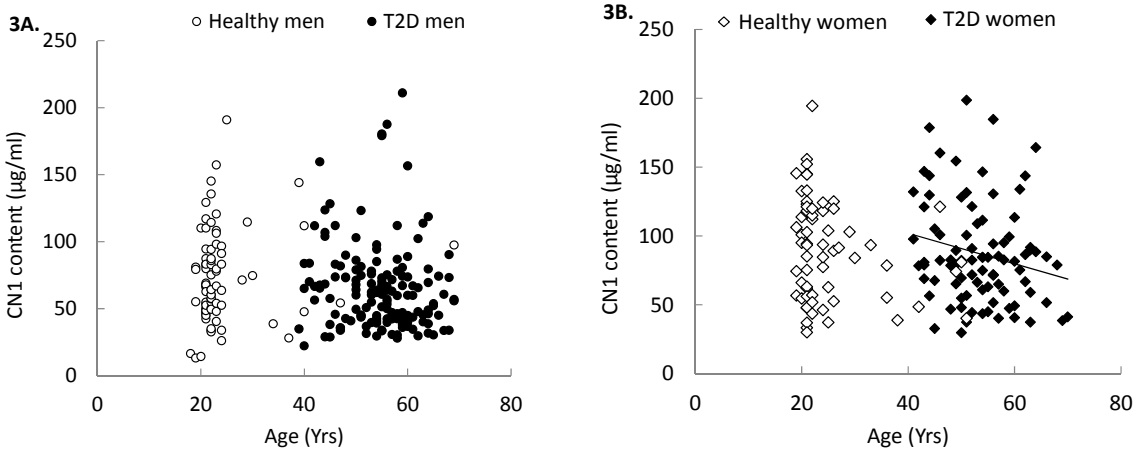


Figure 2. 2A represents the correlation between CN1 content and CN1 activity at baseline for all type 2 diabetic patients (trendline is given), 2B and 2C show the intra-individual variation for respectively CN1 content and CN1 activity over a 6-month period for control subjects only (line of identity is given). Significance level was set at $p<0.05$.

Influence of age, glycemic control and exercise training on CN1 in T2D patients.

Figure 3 represents CN1 content and activity according to age. Here, only correlations are made for the diabetic population as the healthy subjects are all from the same age. In diabetic patients, CN1 protein content is negatively correlated with age in women (r=-210; p=0.049) (figure 3B), but not in men (r=-0.122, p=0.132) (figure 3A). CN1 activity is negatively correlated with age, for both men (r=-0.186; p=0.022) and women separately (r=0.273; p=0.011) (figure 3C and 3D). There is no correlation between HbA1c and CN1 protein content or activity in patients with diabetes type 2 (table 2).



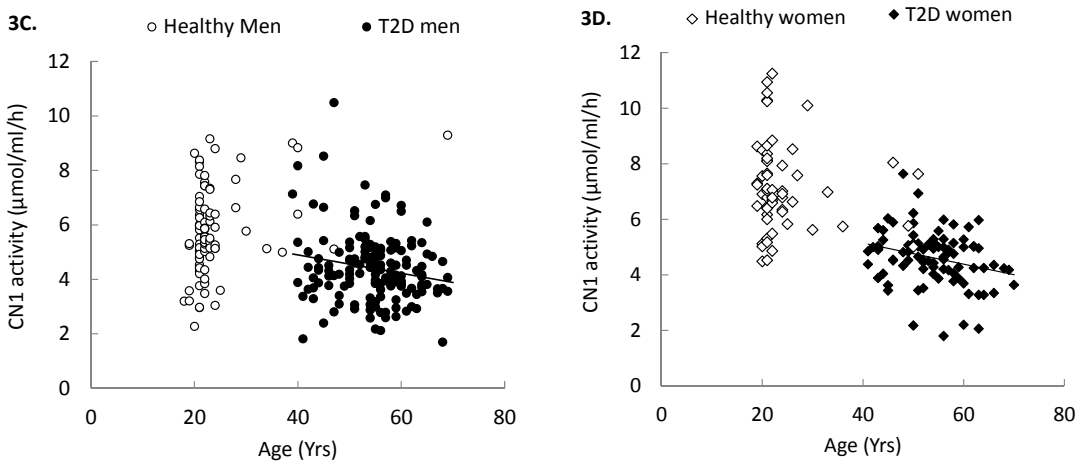


Figure 3. CN1 content (3A and 3B) and activity (3C and 3D) are presented in relation to the age of the subjects, which are shown separately for men (3A and 3C) vs women (3B and 3D) and healthy vs type 2 diabetes (T2D). Significant correlations are given with trendline. Significance level was set at $p < 0.05$.

	CN1 content		CN1 activity	
	Beta-coefficient	p-value	Beta-coefficient	p-value
Glycemic control				
HbA1c	0,036	0,596	-0,007	0,920
Lipid profile				
Triglycerides	0,131	0,055	0,046	0,485
Total cholesterol	0,056	0,412	0,097	0,145
HDL	**-0,153**	**0,020**	**0,131**	**0,038**
LDL	0,027	0,706	0,278	0,782
Total cholesterol/HDL	**0,182**	**0,007**	-0,039	0,555
Blood pressure				
BPS	-0,039	0,563	0,085	0,190
BPD	-0,097	0,146	0,005	0,937

Table 2. Correlations between CN1 content and activity (taken as natural logarithm) and glycemic control, lipid profile and blood pressure, adjusted for age and sex (baseline measurements). Beta-coefficients and p-values are given. Significance level was set at $p<0.05$.

The effect of a 6-month exercise intervention on CN1 could be investigated. Table 3 provides the changes in CN1 protein content and activity, by time and exercise training for 6 months, and adjusted by age, gender and BMI. Neither CN1 protein content, nor CN1 activity was influenced by a 6-month exercise program, irrespectively the type of exercise (neither aerobic or resistance exercise alone, nor the combination of both types).

	Baseline			6 Month			6 Months - Baseline			
	N	Mean	SE	N	Mean	SE	Adjusted Mean	Lower CI	Upper CI	P-value
Mixed models for CN1 content (expressed as µg/ml)										
Control	61	69.07	3.80	59	71.45	4.02	2.32	-1.15	5.79	0.19
Aerobic	59	67.74	3.83	49	69.14	4.04	1.41	-2.44	5.25	0.47
Resistance	62	70.31	3.79	55	72.73	4.05	2.32	-1.33	5.97	0.21
Combined	59	66.95	3.65	57	66.78	3.72	-0.17	-3.88	3.53	0.93
6 Months - Baseline:							**Difference**	**Lower CI**	**Upper CI**	**P-value**
Aerobic vs Control							-0.91	-6.08	4.25	0.73
Resistance vs Control							0.00	-5.04	5.03	1.00
Combined vs Control							-2.50	-7.57	2.57	0.33
Mixed models for CN1 activty (expressed as µmol/ml/h)										
Control	61	4.25	0.15	58	4.40	0.17	0.15	-0.15	0.46	0.32
Aerobic	57	4.26	0.15	48	4.44	0.19	0.19	-0.15	0.52	0.27
Resistance	62	4.54	0.15	54	4.62	0.18	0.07	-0.24	0.39	0.64
Combined	59	4.38	0.15	56	4.27	0.17	-0.11	-0.42	0.21	0.50
6 Months - Baseline:							**Difference**	**Lower CI**	**Upper CI**	**P-value**
Aerobic vs Control							0.03	-0.42	0.48	0.88
Resistance vs Control							-0.08	-0.51	0.36	0.73
Combined vs Control							-0.26	-0.70	0.18	0.24

Table 3. Changes in CN1 protein content and activity, by time and exercise training for 6 months. Results are estimated means from linear mixed-effects models, adjusted for age, gender and BMI. Significance level was set at $p<0.05$.

The pre-post samples of the non-exercise control group demonstrate a good test-retest for CN1 (figure 2B and 2C). The intraclass correlation coefficient (ICC) for CN1 content and activity over a 6-month period in the sedentary diabetic-control group was 0.914 ($p=0.000$) and 0.625 ($p=0.000$), respectively, demonstrating an excellent stability for CN1 content, but only a moderate stability for CN1 activity.

CN1, lipid profile and blood pressure in T2D patients (table 2)

Concerning the lipid profile, the ratio of total cholesterol/HDL was positively correlated with CN1 content ($p<0.01$). Although the relationship was significant in a linear regression as well ($r=0.167$, $p=0.012$), a logarithmic function better met the model ($r=0.182$, $p=0.007$). CN1 activity on the other hand, was not associated with the ratio total cholesterol/HDL. In addition, HDL alone was negatively correlated with CN1 protein content ($r=-0.153$, $p=0.020$), but positively with CN1 activity ($r=0.131$, $p=0.038$). Interestingly, these effects appear to be sex-specific as the correlations are predominantly present for men (CN1 content: total cholesterol/HDL: $r=0.239$, $p=0.002$;

HDL: r=-0.168, p=0.039) (CN1 activity: HDL: r=0.271, p=0.001), but not for women. Finally, blood pressure and CN1 protein content or activity were not correlated with each other.

DISCUSSION

CN1 in healthy versus T2D subjects

It was hypothesized that T2D patients have higher CN1 levels (both content and activity) compared to healthy subjects. This assumption was based on the study of Riedl et al. (2010), who demonstrated that N-glycosylation increases CN1 content and activity in Cos-7 cells. Moreover, they hypothesized that hyperglycemia increases N-glycosylation and thus increases CN1 activity as well, which was confirmed in a small sample of type 1 and 2 diabetic patients compared to healthy subjects.

This study provides evidence that T2D patients are not characterized by elevated CN1 levels. First, no correlation is present between HbA1c and CN1 (content and activity) in diabetic type 2 patients. Second, CN1 content is similar between populations, and CN1 activity is even significantly lower in T2D patients compared to healthy subjects. Moreover, this effect is more pronounced in diabetic women than in men, resulting in a disappearance of the sex difference in CN1 activity which is usually present in healthy subjects. As consequence, CN1 specific activity is lower for T2D patients, and here as well more pronounced in diabetic women (-32%) than in men (-13%). The 'specific' activity is the activity of an enzyme per milligram of total protein (expressed in µmol/min/mg) and is a measure of enzyme quality. Alteration of normal enzyme function is indeed one of the side-effects of chronic hyperglycemia (Brownlee 2005). As mentioned in the introduction, N-glycosylation was hypothesized to increase CN1 activity, however it is also known that glycation (non-enzymatic glycosylation) has detrimental effects on proteins (and thus also enzyme activity) by disrupting molecular conformation, possibly partly explaining the observed decrease in specific activity (Aronson 2008). The observed decrease in CN1 activity in a diabetic population compared to healthy controls could be interpreted as protective because of higher bioavailability of CN1 substrates, carnosine and anserine. Indeed, we have demonstrated in the introduction that a polymorphism in the serum carnosinase gene (CNDP1), encoding for low CN1 activity, is associated with reduced risk of diabetic nephropathy (Janssen 2005; Zhu 2013).

We suggest that this observation is specific for a diabetic population, but caution is warranted as the age range was different between the two populations. However, to our

opinion this age difference does not explain a decrease in CN1 as no (or a slight) increase was reported previously in literature from the age of 20 years until the age of 50 years (Lenney 1982; Peters 2010). Moreover, the observed decrease in CN1 activity with age in T2D patients (age range: 39 – 70 yrs) is perhaps an epiphenomenon of increased duration of diabetes (data lacking).

It was previously reported that CN1 activity is greatly determined by its content in healthy adult individuals as demonstrated by Adelman et al. (r=0.79) and Everaert et al. (r=0.58). However, this study demonstrates lower correlations in both populations. Healthy and diabetic women and healthy men display similar correlations (respectively r=0.305 with p=0.020, r=0.345 with p=0.001 and r=0.469 with p<0.001), whereas a dissociation between content and activity is present in diabetic men (r=0.149, p=0.067). Clearly, the correlation between CN1 activity and CN1 content is not so strong as it was previously presented, especially in the diabetic men. Therefore, it is necessary to further search for factors that specifically influence CN1 activity (without a change in CN1 content). For the moment, only two possibilities have been suggested; allosteric conformations of CN1 in children and adults (Peters 2010) and competitive inhibition by homocarnosine and anserine (Peters 2011b).

Effect exercise training on CN1 in T2D patients

We hypothesized that exercise training (aerobic, resistance or combined exercise) would suppress CN1 content and activity, but this could not be confirmed. The hypothesis was based on 2 observations. First, Baguet et al. (2014) reported lower CN1 levels (both content and activity) in elite athletes, involved in high-intensity exercise, compared to untrained controls (data own lab). Second, Riedl et al. (2010) showed elevated CN1 activity levels in diabetic patients, suggesting that exercise training, aiming for ameliorating insulin sensitivity and thereby lowering hyperglycemia, decreases CN1 activity levels. Concerning the latter, we have now demonstrated that T2D patients are not characterized by higher CN1 activity levels and therefore it is a rather logical consequence that lowering hyperglycemia (through exercise) has no effect. Nevertheless, we can exclude that exercise training (according to either one of the two theories) lowers CN1 activity.

Low CN1 content/activity associated with lipid profile and blood pressure?

Several studies have recently brought the attention to the protective effect of carnosine administration on dyslipidemia (lipid profile in plasma (Aldini 2011; Mong 2011), cholesterol levels in heart and liver (Lee 2005; Mong 2011), atherosclerosis (Menini 2012)) and blood pressure (Aldini 2011) in rodents. Our hypothesis that diabetic patients with the lowest CN1 levels, should have the highest circulating carnosine levels upon dietary ingestion and therefore should have the most healthy lipid profile, could not be convincingly accepted. Low CN1 content is associated with high HDL levels (p=0.020) and a low ratio total cholesterol/HDL (p=0.007), which indeed confirms our hypothesis, since both associations represent a beneficial lipid profile. When both sexes are analyzed separately, this pattern remains present in the men (HDL: B=-0.168, p=0.039; total cholesterol/HDL: B= 0.239, p=0.002), but not significantly in women (HDL: B=-0.119, p=0.265; total cholesterol/HDL: B=0.092, p=0.402). Yet, when looking at CN1 activity, our hypothesis must be rejected. Only HDL is significantly correlated with CN1 activity, although in the opposite way (B=0.131, p=0.038) and only in the men (B=0.271, p=0.001). As already mentioned above, there appears to be a dissociation between CN1 activity and CN1 content in diabetic type 2 patients, predominantly in the men.

Mong et al. (2011) is the first in providing an explanation for this possible lipid lowering effect. His group demonstrated that carnosine supplementation markedly lowered lipid accumulation in circulation, liver and adipose tissue in high fat diet mice via diminishing the mRNA expression of sterol regulatory element binding proteins (SREBPs). SREBPs are important transcription factors responsible for fatty acid and cholesterol metabolism (Shimano 1999), in which SREBP-1c is more effective in modulating the expression of genes involved in fatty acid synthesis, whereas SREBP-2 is more predominant in mediating genes associated with cholesterol synthesis (Shimomura 1997; Horton 1998).

Conclusion

To summarize, we provided evidence that diabetic patients are not characterized by elevated CN1 levels. While CN1 content was equal between healthy subjects and T2D patients, CN1 activity was even lowered in T2D patients. Moreover, there was no relationship between CN1 (content and activity) and HbA1c in diabetic patients, as consequence a 6-month exercise program, aiming to lower HbA1c, could not suppress CN1. Finally, CN1 content and activity have contrasting associations with markers of dyslipidemia.

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Part III

General Discussion

1. CARNOSINE-CARNOSINASE SYSTEM IN A DIABETIC STATE

From literature it was suggested that both muscle carnosine content and serum carnosinase (CN1) are possibly altered in a diabetic state. In this first chapter we will provide an answer based on **studies 1, 3, 4** and **5**.

1.1. Diabetes associated changes in muscle carnosine content

In part I, it was suggested that carnosine levels in tissues are possibly decreased in a diabetic state. Some rodent studies demonstrate a decrease in diabetic liver, retina and kidneys, however these findings are not confirmed by others (they demonstrated a decrease, although not significantly) (Riedl 2011; Pfister 2011; Mong 2011). Concerning carnosine content in skeletal muscle, only two studies exist and they are contradictive (Gualano et al. reporting a decrease and Skrikanthan et al. reporting a increase). The working hypothesis behind this 'possible' decrease is the fact that carnosine can quench RCS (Aldini 2011), possibly followed by a clearance from the muscle as hypothesized by Hipkiss and coworkers (Hipkiss 2000a).

In **study 4** we provided evidence that muscle HCDs increase with progressive glucose tolerance, which was demonstrated by both an interventional study (in rodents) and a cross-sectional study (in humans). In the rodent study, muscle carnosine increased with ~30% in the most drastic overfed, glucose-intolerant rodent model (high-fat diet compared to control diet) ($p<0.05$). Anserine was not significantly increased (+16%), but the total amount of HCD tended to increase (+21%, $p<0.1$). In the human study, muscle carnosine of prediabetic and diabetic type 2 patients increased with 30% ($p<0.05$) and 39% ($p<0.05$), respectively, compared to the healthy lean subjects.

However, we cannot deny the data of Gualano et al. (2012) (a joint project) reporting a 45% decrease in gastrocnemius muscle of type 2 diabetic patients compared to healthy controls. Therefore, we here provide **a working model** with diabetes-related factors possibly influencing baseline muscle carnosine content, as presented in figure 1. Chapter 1.1.1. will focus on diabetes-related factors explaining a possible decrease in muscle carnosine content, whereas chapter 1.1.2. will focus on the factors explaining a possible

increase in muscle carnosine content. According to the dominance of certain factors, muscle carnosine content will increase, decrease or remain equal.

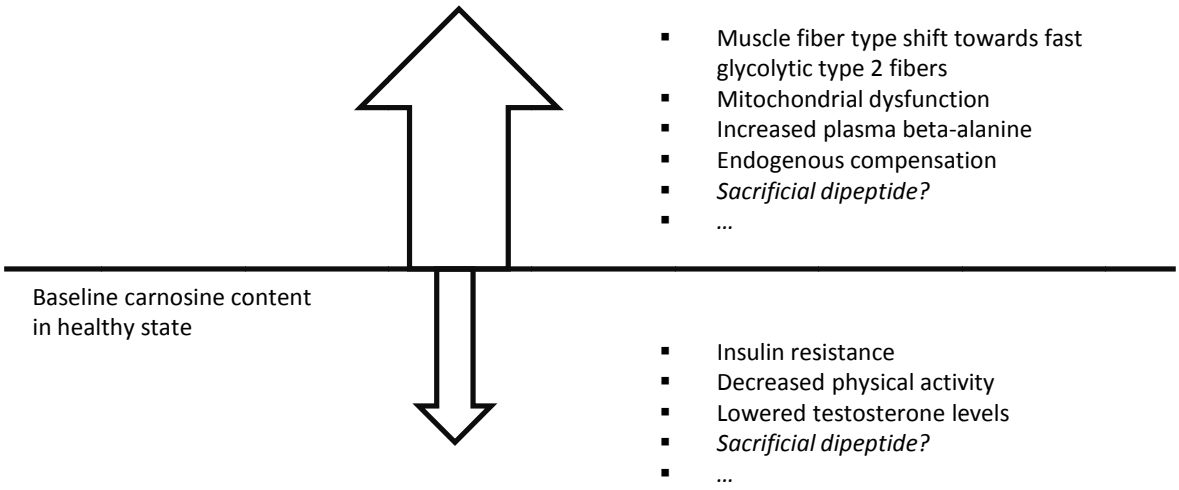


Figure 1. Possible diabetes-associated factors influencing baseline muscle carnosine content.

1.1.1. **Factors decreasing muscle carnosine content**

1.1.1.1. Sacrificial dipeptide

All forms of diabetes are associated with glycoxidative and lipidoxidative stress, manifesting itself by the presence of deleterious species such as RCS, AGEs and ALEs (Brownlee 2001; Wellen 2005; Vistoli 2013). Interestingly, carnosine is believed to act as a self-sacrificial dipeptide predominantly towards RCS, possibly resulting in decreased tissue carnosine levels. However, before this work only one study existed about the *in vivo* quenching ability (Aldini 2011).

Study 3 confirmed the ability of carnosine to quench RCS such as HNE *in vivo*. High-fat diet rats receiving carnosine in their drinking water demonstrated Carn-HNE adducts in urine and plasma (this is the first study detecting Carn-HNE adducts in plasma), whereas these adducts were absent in the non-supplemented groups. However, these results point to a systemic elimination of carnosine-HNE adducts, and not towards the quenching and elimination from muscle.

Interestingly, Baba et al. (2013) confirmed very recently the presence of these Carn-HNE adducts in skeletal muscle of C57BL/6 mice. Moreover, they showed for the first time that these adducts, a.k.a. carnosine propanals, have still some reactive activities but that these propanals are in a next step reduced (detoxified) by aldose reductase, forming carnosine propanols. These species carry no reactive properties any longer.

However, a crucial link is still missing. How are these adducts actually cleared from the skeletal muscle? Up to now, there is still no evidence for a regulated degradation mechanism, except for the theory of Hipkiss et al. (2000), which hypothesizes proteolysis of carnosine-protein-carbonyl adducts (carnosinylated proteins) (figure 10, part I), however there is no clear evidence provided to support such a mechanism.

In this work, we could confirm the ability of carnosine to quench RCS such as HNE in urine and plasma of high-fat diet rats. In addition, these adducts are recently confirmed in skeletal muscle. However, up to now, no evidence exists for a possible degradation pathway of these adducts.

1.1.1.2. Insulin resistance

Skeletal muscle insulin resistance is a typical characteristic for diabetes type 2 (DeFronzo 1991; Kruszynska 1996; Kahn 2003; DeFronzo 2009), but it plays also a role in type 1 diabetes disease more than is commonly recognized (DeFronzo 1982; Bergman 2012; Donga 2013).

In **study 1** we demonstrated that meal and beta-alanine co-ingestion stimulates muscle carnosine loading, suggesting that insulin plays a role. Although this study was more designed to study the 'loading' aspect, this mechanism can probably also affect baseline levels. First, we will discuss our results from the loading study (study 1), to extrapolate these data thereafter to baseline carnosine levels in a diabetic state.

The way how insulin increases muscle carnosine loading can be explained by two possible mechanisms. First, it has been shown that insulin stimulates the Na^+/K^+ pump in skeletal muscle, which increases extracellular Na^+ availability in circulation (Clausen 2003). In turn, increased extracellular Na^+ availability stimulates the sodium dependent beta-alanine transporter TauT. So, it is likely that stimulating TauT (by meal) at the moment of beta-

alaninemia (by beta-alanine ingestion), results in higher beta-alanine transport into the muscle and thus more carnosine synthesis. This mechanism is illustrated in figure 2. Another possible mechanism is the ability of insulin to stimulate muscle blood flow (Baron 1994), and thus increasing the beta-alanine availability for the muscle.

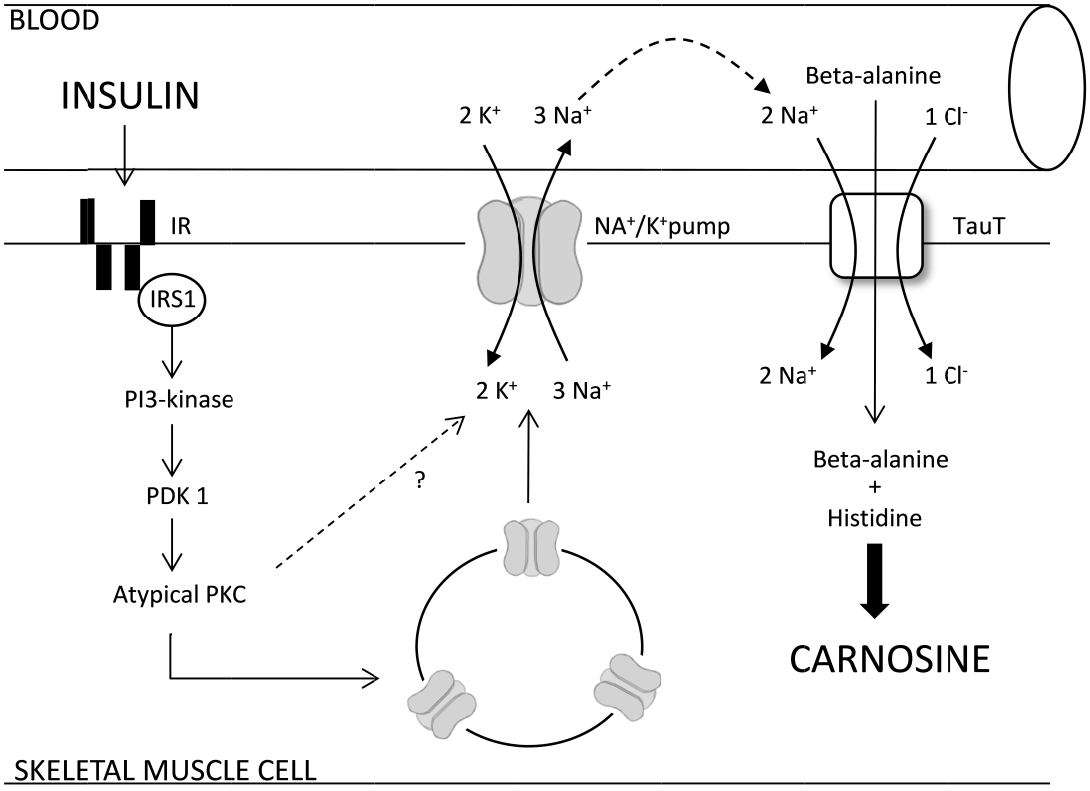


Figure 2. Schematic representation of how insulin can stimulate beta-alanine uptake through Na+/K+ pump stimulation. IR: insulin receptor, IRS1: insulin-receptor substrate-1.

Both mechanisms can go hand in hand and can also explain why the effect was only significantly present in soleus (a more oxidative muscle) and not in gastrocnemius (a more glycolytic muscle). First, Lavoie et al. (1996) demonstrated that insulin-induced translocation of Na^+/K^+ ATPase subunits to plasma membrane is restricted to oxidative fibers (Lavoie 1996). Second, Utriainen and coworkers (1997) has demonstrated that insulin increases muscle blood flow preferentially to areas with high rates of glucose uptake, i.e. the more insulin-sensitive type 1 fibers (Utriainen 1997).

Coming to our point, both insulin-dependent mechanisms can attribute to a decrease in muscle carnosine content in a diabetic state. First, diabetes and insulin resistance is associated with downregulation of the Na^+/K^+ pump content (Kjeldsen 1987; Djurhuus

2001; Clausen 2003; Galuska 2009), which is unfavorable for the sodium-dependent beta-alanine transporter TauT and thus beta-alanine uptake can be declined. Second, insulin resistant patients are characterized by lower muscle capillary density (Lillioja 1987; Marin 1994; Rattigan 2007), which decreases beta-alanine availability for the muscle, again possibly blunting beta-alanine uptake. Off course, it can be questioned whether these factors play a role in situations where no beta-alanine is exogenously obtained (as in our rodent models, study 4).

Conclusion: **We demonstrated in study 1 that meal and beta-alanine co-ingestion enhances muscle carnosine loading, suggesting that insulin plays a role. Extrapolating these data to non-loading conditions, insulin resistance could have a lowering effect on baseline levels.**

1.1.1.3. Decreased physical activity

Diabetic patients are often (not always) characterized by a sedentary lifestyle (Myers 2003; Hu 2003). The way how decreased physical activity can possibly lower muscle carnosine content is actually closely related to the previous factor (insulin resistance), as these factors share similar mechanisms. Na^+/K^+ pump activity and muscle blood flow are influenced by both insulin and contraction (Benziane 2008), like it is also the case for the glucose transporter GLUT4. Contraction (acute) and chronic exercise can respectively acutely and chronically increase muscle blood flow. In addition, contraction stimulates the Na^+/K^+ ATPase activity in skeletal muscle, independently from insulin stimulation (Benziane 2008).

In line with this, our own lab recently demonstrated that carnosine loading is more pronounced in trained versus untrained muscles of athletes (Bex 2013). Muscle carnosine loading was compared between the arms and the legs of kayakers (predominantly using their arms) and cyclists (predominantly using their legs). Muscle carnosine loading was 80% higher in the arms of the kayakers than in the legs, and the other way around, loading was higher in the legs than in the arms of the cyclers.

Similar as above, we can question whether this factor can contribute in a situation where no exogenous beta-alanine is taken (as in study 4).

1.1.1.4. Lowered testosterone levels

Low testosterone levels are common in men with obesity and type 2 diabetes and may be associated with insulin resistance (Grossmann 2008; Grossmann 2013). In addition, muscle carnosine levels have been shown to be negatively modified by orchidectomy in male mice (Penafiel 2004; Everaert 2013). Although Everaert et al. (2011) demonstrated that muscle carnosine levels are not associated with total testosterone and free testosterone plasma levels in healthy subjects, it is possible that markedly lowers levels (as it is the case in obese and diabetic men) can result in decreased muscle carnosine levels.

1.1.2. Factors increasing muscle carnosine content

1.1.2.1. Muscle fiber type shift towards type II fibers

Several studies have shown that obesity and diabetes (both type 1 and type 2) are associated with an increased proportion of fast glycolytic type II fibers and a reduced percentage of slow type I fibers compared with lean healthy subjects (figure 3) (Tanner 2002; Oberbach 2006; Fritzsche 2008; Stuart 2013). From part I it was clear that (fast glycolytic) type II muscle fibers possess more (almost a double) carnosine compared to oxidative type I fibers (Harris 1998; Kendrick 2009). This was confirmed on muscle level in both our human (study 1 and 2) and rodent studies (studies 3 and 4) of this work. Therefore, it is possible that fiber type shift is an important factor contributing to an increase in carnosine concentration in a diabetic state.

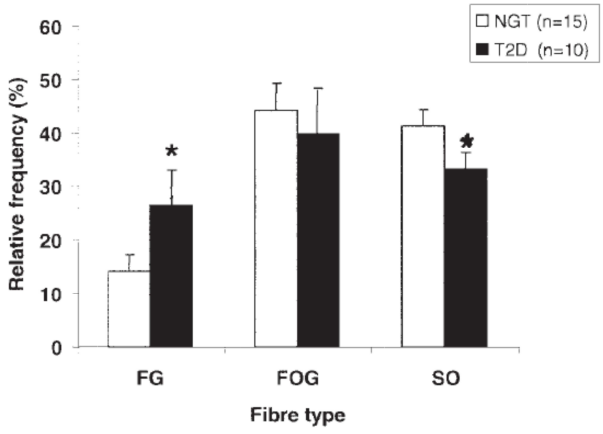


Figure 3: Skeletal muscle fiber type distribution in healthy subjects with normal glucose tolerance (NGT, white bars) and age- and BMI-matched subjects with type 2 diabetes (T2D, black bars). *p<0.05 for NGT vs. T2D (Oberbach 2006).

However, two important remarks must be made. First, cause and effect are under debate. It is suggested that a greater proportion of fast glycolytic type II fibers increases the susceptibility for obesity and diabetes, making fiber type a cause and not an effect (Abou 1992; Tanner 2002). Abou et al. (1992) have shown a relationship between the susceptibility to high-fat diet-induced obesity and skeletal muscle fiber type in rats. Obesity-resistant rats had a significantly higher proportion of type I muscle fibers than obesity-prone rats both before (determined from a muscle biopsy) and after the 4-week high-fat diet period (figure 4).

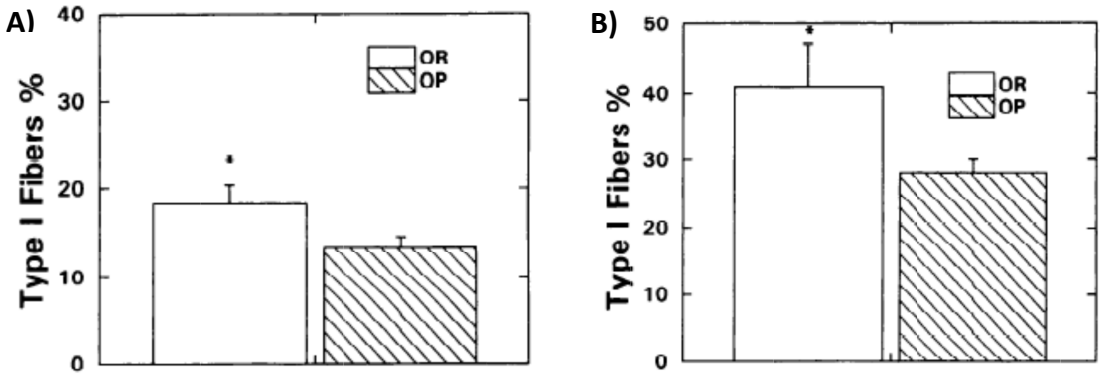


Figure 4: Proportion of type I muscle fibers found in medial head of gastrocnemius muscle for obesity-prone (OP) and obesity-resistant (OR) rats. A) results obtained from muscle biopsies taken before high-fat diet. B) results obtained from muscle removed at end of high-fat diet feeding period. * Significant ($P<0.05$) difference between groups (Abou 1992).

Second, treatment of lean animals with a high-fat diet is followed by muscle fiber alterations in favor of the oxidative metabolism including increased proportions of type I fibers in skeletal muscle, in an attempt to remove the overload of fat delivery (Abou 1992; Shortreed 2009). Indeed, figure 4b (after high-fat diet) displays increased % type I fibers, compared to figure 4b (before the high-fat diet).

These apparently opposing findings may be the sequential outcome of a complex mechanism that regulates excess of fat availability, being different for the initial phase compared to the long-term phase and being different for oxidative versus glycolytic muscles. In line with this, Yasuda et al. demonstrated a higher % of type I fibers (but a lower % of type IIA fibers) in soleus muscle of 5- and 9-week-old Goto-Kakizaki rats (non-obese diabetic rats). On the other hand, a remarkable increase was observed in type IIB fibers (and a lower % of type I and IIA fibers) in plantaris muscle of 9- and 20-week-old Goto-Kakizaki rats.

Nevertheless, we can conclude that fiber type can explain an increase in muscle carnosine in obese and diabetic individuals, but it is questionable whether this was responsible for an increase in our high-fat rodent models (study 4).

1.1.2.2. Mitochondrial dysfunction

It became clear from **study 1** that during beta-alanine supplementation, the major part (> 90%) is oxidized/metabolized in the body. There are some indications that the mitochondrial enzymes 'malonic semialdehyde dehydrogenase, MSAD' and 'beta-alanine transaminase, ABAT' are taking part in the oxidation of beta-alanine (Pollitt 1985; Everaert 2013). In addition, Lefort et al. (2010) has shown that single mitochondria, isolated from skeletal muscle in obese patients, have lower amino acid-metabolizing enzymes such as MSAD (Lefort 2010). Moreover, Pollitt et al. reported that a deficiency in MSAD results in excessive excretion of beta-alanine (Pollitt 1985). If this would be similar with beta-alanine in skeletal muscle, more beta-alanine would be available in skeletal muscle to be converted to carnosine/anserine.

1.1.2.3. Sacrificial dipeptide

Strangely enough, investigating the 'sacrificial theory' from another perspective can lead to an increase of tissue carnosine rather than a decrease. As mentioned above Baba et al. (2013) published very recently a crucial paper, by demonstrating that carnosine-propanals (these are adducts of carnosine with reactive carbonyl species such as HNE and acrolein) are reduced/detoxified by the enzyme aldose reductase (AR) to carnosine-propanols (Baba 2013). In addition, AR-null mice (mice lacking the enzyme AR) had higher levels of carnosine-propanals in skeletal muscle because these conjugates were not reduced. Interestingly, in hyperglycemic conditions, the enzyme aldose reductase is predominantly used to eliminate the excess of glucose, resulting in less availability of AR for reducing the carnosine-propanals, and thus an accumulation of these conjugates.

On the other hand, it can be also argued that the sacrificial theory has no effect at all, because 1) no degradation pathway for these carnosine conjugates is established, 2) the total amount of carnosine, carnosine-propanals and carnosine-propanols probably does

not change and 3) the methods measuring carnosine (HPLC and ^{1}H-MRS) probably detect all forms.

1.1.2.4. Increased plasma beta-alanine availability

Very recently Zhou et al. (2013) evaluated plasma amino acid differences between healthy individuals (lean and obese) and diabetic type 2 individuals (lean and obese)(Zhou 2013). In general, fasting plasma beta-alanine was 18% higher in diabetic group (25 µM/l) compared to the control group (21.1µM). Interestingly, the biggest difference was found between the lean healthy (16.9 µM/l) and the lean diabetic (24.8 µM) subjects, whereas the difference was less pronounced between the healthy obese (22.2 µM/l) and diabetic obese (25.1 µM/l) subjects, suggesting that diabetes rather than obesity is responsible for this increase.

Comparing these results to our own data from **study 1** in which plasma beta-alanine was followed throughout the day in 4 subjects, it is remarkable that Zhou and colleagues found relatively high fasting plasma beta-alanine levels in all subjects, whereas we could often not detect beta-alanine in fasted state (detection limit: 7 µM).

1.1.2.5. Endogenous compensation

In general, the body is equipped with several endogenous protective systems, activated once homeostasis in the normal healthy body is disturbed (principle of pursuing homeostasis). For example, in response to the supplementary production of ROS during exercise, the antioxidant system will be activated, resulting in an improved antioxidant status of the individual (Liu 2000; Finaud 2006).

Related to this work, it could be possible that a kind of compensation mechanism is activated by factors responsible for a decrease in muscle carnosine (decreased Na^{+}/K^{+} pump activity, decreased muscle blood flow, the quenching of increased ROS,…). A compensation mechanism regarding muscle carnosine synthase has been demonstrated by our own lab (Everaert 2013). Muscle carnosine decreased (-47%) after orchidectomy in mice, whereby a compensatory increase in carnosine synthase mRNA levels (a 2-fold) occurred.

1.1.3. **Conclusion: the balance between decreasing and increasing factors**

In **study 4** we could demonstrate, both in rodents (interventional) and in humans (cross-sectional), that muscle HCDs increase with progressive glucose intolerance. However, we cannot deny the data of Gualano et al. (2012) (a joint project) reporting a 45% decrease in gastrocnemius muscle of type 2 diabetic patients compared to healthy controls. The different conclusions between our data and the data of Gualano et al. (2012) can possibly be explained by a difference in diabetes-stage. The animals tested in study 4 were only slightly insulin resistant and our T2D patients were mild diabetic as they were diet-treated only. On the other hand, the T2D patients of Gualano et al. (2012) were on oral hypoglycemic medication, which is a phase further in diabetes disease compared to our subjects. Thus, it is possible that insulin resistance (a factor responsible for decreasing muscle carnosine) was not yet very prominent in our T2D patients and thus a factor of minor importance. Perhaps, insulin resistance can become a more dominant factor (in determining muscle carnosine content in a diabetic state) in more severe diabetes.

Therefore, we provided a working model that muscle carnosine in a diabetic state is a balance between diabetes-related factors responsible for an increase and factors responsible for a decrease. Depending on the factors dominating in a specific situation, muscle carnosine will be increased, decreased or remain equal.

1.2. Serum carnosinase in a diabetic population

In the introduction (part I) it was suggested that CN1 may play a role in the development of diabetes or diabetes-related complications (Janssen 2005; Sauerhofer 2007; Riedl 2010). Briefly, low CN1 is assumed to be protective because the bioavailability of its substrates carnosine and anserine in circulation would be higher, as they would be less rapidly hydrolyzed by CN1. Thus, more protection against ROS, RCS, AGEs and ALEs can be provided by carnosine and anserine, resulting in a decreased risk of diabetic related complications.

In relation to diabetes, it is speculated that *hyperglycemia* increases CN1 content and activity. Riedl et al. (2010) demonstrated in transfected cells that N-glycosylation is an important determinant for CN1 content and activity. The authors further extrapolated this observation to the assumption that hyperglycemia would increase CN1 activity, by inducing N-glycosylation (see working mechanism in part I). Indeed, their hypothesis was confirmed as they demonstrated that 11 diabetic patients with the 5-5 genotype had ~25% higher CN1 activity levels compared to 15 homozygous healthy controls. From the data above, we expected to see a positive correlation between HbA1c (a marker of hyperglycemia over the past 2-3 months) and CN1 content/activity in a large cohort of diabetic type 2 patients (**study 5)**, but this expectation was not fulfilled (figure 6).

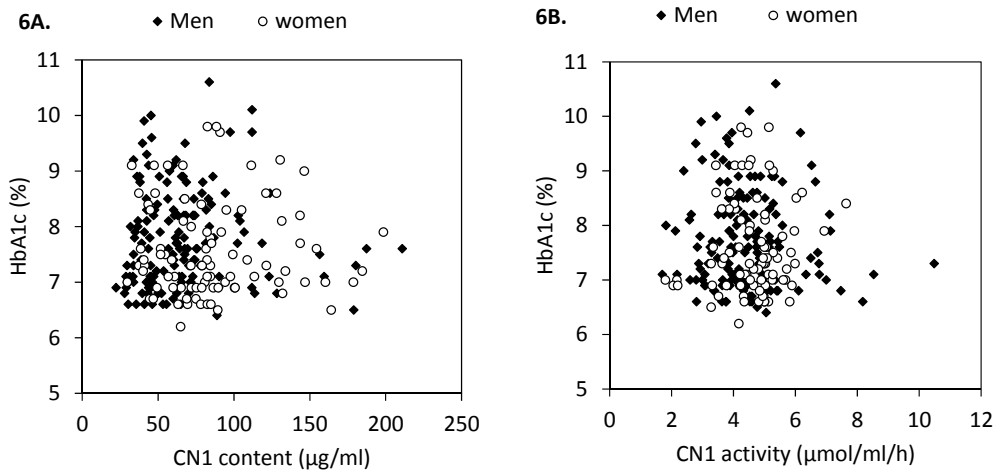


Figure 6. The correlation between HbA1c and CN1 content (A) and CN1 activity (B). No significant correlations are reported.

Moreover, a comparison was also made with a healthy control group. CN1 content was similar between populations and CN1 activity was even significantly lower in type 2 diabetic patients compared to healthy subjects. As consequence, CN1 specific activity is lower for diabetic type 2 patients. Therefore, we suggested in study 5 that CN1 activity in a diabetic state is probably not only influenced by N-glycosylation (stimulating CN1 activity) (Riedl 2010), but also by glycation (decreasing CN1 activity by molecular disruption). Glycation of enzymes, leading to decreased activity, has already been reported previously (Beranek 2001; Bousova 2005a; Bousova 2005b; Aronson 2008).

Caution remains warranted as the control group is not matched for CTG genotype, age and BMI. To our opinion the age difference can not be responsible for a difference between populations as no (or a slight) increase was reported previously in healthy subjects from the age of 20 years until the age of 50 years. The observed decrease in CN1 activity with age in diabetic type 2 patients (age range: 39 – 70 years) is perhaps an epiphenomenon of increased duration of diabetes (data lacking). Nevertheless, a comparison with a correct healthy control group is necessary.

In a next step, we explored whether CN1 is stable over *time* (6-months) and whether *exercise training* (endurance, resistance or the combination), aiming for decreasing HbA1c, influences CN1. When looking at the intraclass correlation coefficients for CN1 content and activity over a 6-month period in the diabetic control group (non-exercise), these were respectively 0.914 ($p<0.001$) and 0.625 ($p<0.001$), demonstrating an excellent stability for CN1 content, but a rather moderate stability for CN1 activity. In addition, irrespectively the kind of exercise program followed, CN1 activity and content did not significantly change, although the HbA1c significantly decreased with 0.90%, 0.43%, 0.30% in the respectively exercise groups: combined, aerobic or resistance exercise training. However, the absence of an exercise effect is not surprising as we demonstrated that diabetic patients do not have elevated CN1 levels.

Finally, we hypothesized that diabetic patients with low CN1 levels, should have the most healthy lipid profile. This hypothesis was based on the theory that individuals with low CN1 levels should have the highest circulating carnosine levels upon dietary ingestion, and thus more carnosine would be available in plasma to exert its lipid-lowering effects

(part I). However, our hypothesis could not be accepted. Only in the men, CN1 content was negatively correlated with HDL (p=0.039) and positively with the ratio total cholesterol/HDL (p=0.002), which indeed confirmed our hypothesis. However, correlations were the other way around for CN1 activity. Nevertheless, future research must incorporate lipid-lowering medication (if taken) into the analysis, as this can be a disturbing factor.

To summarize, we hypothesized that exercise training or a better glycemic control could suppress elevated CN1 levels possibly present in diabetic patients. However, we provided evidence that diabetic patients are not characterized by elevated CN1 levels. While CN1 content was equal between healthy subjects and T2D patients, CN1 activity was even lower in T2D patients. Moreover, there was no relationship between CN1 (content and activity) and HbA1c in T2D patients. In addition, CN1 content and activity are stable over a 6-month period and not influenced by chronic exercise training. And finally, CN1 content and activity have contrasting associations with markers of dyslipidemia.

2. NET MUSCLE CARNOSINE TURNOVER FROM A BROADER PERSPECTIVE.

In this chapter, net muscle carnosine turnover is observed from a broader perspective and discussed for four different phases: muscle carnosine at baseline, during loading, washout and maintenance.

2.1. Net muscle carnosine turnover at baseline

As discussed in **study 2** (part II), net muscle carnosine turnover is a balance between carnosine synthesis and degradation. At baseline, muscle carnosine synthesis and degradation are in a steady equilibrium and predominantly determined by fiber type, age and sex. The role of dietary beta-alanine intake (by meat and fish intake) is up to now not really clear. As we demonstrated in this work, a diabetic state can change this steady equilibrium (chapter I, part III).

2.2. Net muscle carnosine turnover during loading

During beta-alanine supplementation (loading), carnosine synthesis will be elevated because of increased precursor availability and thus net turnover will be strongly positive. There is a **large inter-individual variability** concerning this loading efficiency, which is demonstrated in **study 1.** The highest increase (3.27 mM) was 8 times higher than the lowest increase (0.40 mM) after a supplementation period of 3.2 gr beta-alanine per day for 6-7 weeks.

Study 1 and 2 searched for **determinants** of muscle carnosine loading. Only 1 significant determinant was found, i.e. meal timing. Taking beta-alanine at the start of the meal results in a higher absolute (0.58 mM) and relative (23%) increase compared to taking beta-alanine in between the meals in soleus muscle. On the other hand, muscle fiber type, gender, supplementation form, body weight, fat-free mass and baseline carnosine content did not influence the *absolute* increase. It remains however to be determined whether the higher *relative* increases in women and lighter individuals, which are actually a consequence of the fact that these individuals start with lower baseline levels, are relevant from a practical point of view. As discussed in paper 2, it has already been reported that there is a correlation between the relative change in carnosine and

performance enhancement, but perhaps this observation is secondary to or a result of an absolute increase in muscle carnosine.

Very recently, our own lab found that carnosine loading is more pronounced in trained versus untrained muscles (Bex 2013), which appears to be the most dominant determinant. For example, arm muscles (m. deltoideus) from kayakers had a 80% higher absolute increase than the leg muscles (m. soleus + m. gastrocnemius) after a supplementation period of 6.4g beta-alanine per day for 3 weeks, whereas the opposite pattern was observed in the cyclists. As said, this difference can be attributed to an acute/chronic increase in blood flow, acute/chronic adaptation of transporters and enzymes involved in carnosine synthesis and acute/chronic increase in Na^+/K^+ pump activity. In line with this, it can be assumed that muscle carnosine loading would be less efficient in sedentary and unfit diabetic individuals, exhibiting decreased muscle blood flow and Na^+/K^+ pump activity (cfr supra).

Although the different loading protocols (discussed in study 1 and 2) have proven their ergogenic relevance, caution must remain present as the majority (> 90%) of the ingested beta-alanine has **an unknown metabolic fate**. We have demonstrated in study 1 that only ~3% of chronically ingested beta-alanine is incorporated into muscle carnosine and only a similar amount is lost through urinary excretion. When comparing this efficiency to the efficiently of creatine supplementation, another popular nutritional supplement, this is very low (figure 7). Figure 7 compares the efficiency of beta-alanine and creatine supplementation, each with and without coingestion of macronutrients (MN).

Notwithstanding the fact that the accumulation efficiency is higher when beta-alanine is ingested with a meal (~20% higher) compared to ingestion in between the meals and the fact that trained muscle are more efficient than untrained muscles (~80% higher), the % ingested beta-alanine accumulated in skeletal muscle remains remarkably low (figure 7). Therefore, the question raises ‘where does all the beta-alanine go?’ Two possibilities can be put forward.

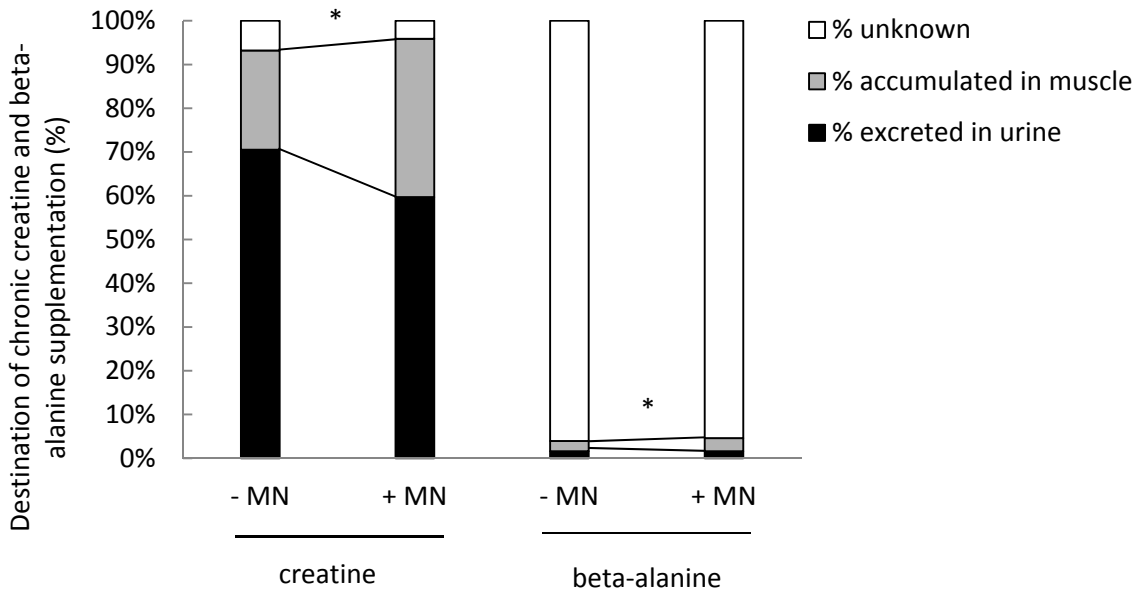


Figure 7. Comparison of the destination of chronic beta-alanine supplementation (with '+MN' or without '- MN' macronutrients) with the destination of chronic creatine supplementation (- MN and + MN). * $p<0.05$, a significance increase in muscle accumulation +MN vs –MN. (Steenge 2000)

First, beta-alanine can be converted into carnosine in other (non-muscle) tissues. However, as the presence of carnosine and carnosine synthase in non-muscle tissues is much lower than in muscle, this pathway has probably little quantitative significance. Therefore, a major part of the ingested beta-alanine is likely metabolized somewhere else (*second* possibility). ABAT (beta-alanine transaminase, also known as 4-aminobutyrate aminotransferase) is an important enzyme in the oxidation of beta-alanine. It can convert beta-alanine into malonate semi-aldehyde, which be decarboxylated to acetaldehyde and further converted to acetyl-CoA, to finally enter the citric acid cycle. De Biase et al. (1995) demonstrated that the expression of ABAT is predominantly present in liver, but high amounts are also present in pancreas, brain and kidney in human tissues (De Biase 1995). Our lab recently focused on this enzyme in skeletal muscle and demonstrated its mRNA expression in rodent skeletal muscle and showed an upregulation upon beta-alanine supplementation (Everaert 2013). It remains to be determined to what extend ABAT can contribute to the 'unknown metabolic fate', and whether or not other enzymes are involved.

2.3. Net muscle carnosine turnover during wash-out and maintenance

When daily beta-alanine intake over a prolonged period (loading) is terminated, a slow degradation process takes place, reaching pre-supplementation levels in 6-20 weeks (Baguet 2009; Stellingwerff 2012). This wash-out can be attributed to an increased elimination rate, induced by increased muscle carnosine levels through prolonged supplementation (calculated from Hill 2007b, discussion study 2).

Therefore, to keep muscle carnosine at a constant elevated level a maintenance dose is recommended to counteract the increased elimination rate. **Study 2** is the first study in establishing a suitable maintenance dose of ~1.2g beta-alanine/day to keep muscle carnosine content elevated at 30-50% above baseline. Taking together the current knowledge, athletes must be advised to phase their beta-alanine intake towards the competition as represented in figure 8.

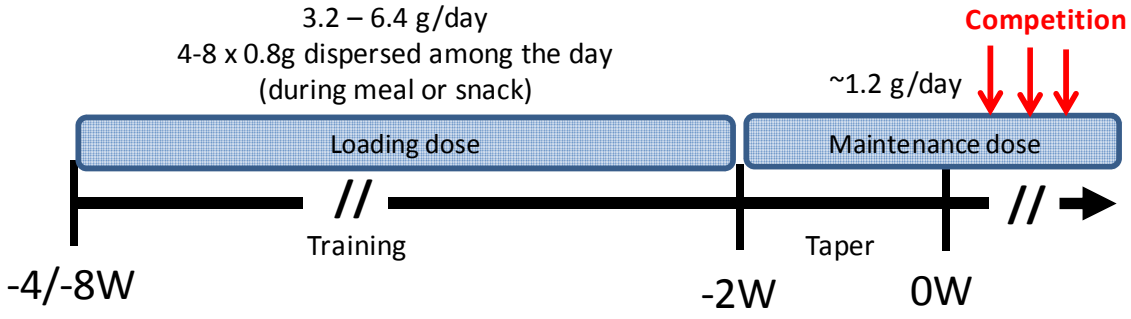


Figure 8. Current advice of beta-alanine supplementation prior to peak performance or competition period.

Although it can be argued why athletes would not continue to take a loading dose on the off-chance that carnosine levels continue to increase, we would currently disadvise non-stop supplementation because of the following arguments: 1) physiological effects of long-term supplementation (> 12 w) with high doses are unknown, 2) we must keep in mind that 90% of ingested beta-alanine is unaccounted for and 3) no data exists about the acute effects of high doses of beta-alanine on performance (negative or positive).

3. EFFECTS OF CARNOSINE SUPPLEMENTATION

3.1. Site of action: systemic effect or tissue effect

As said in the introduction, the role of carnosine must be explored from two approaches. The first one, investigating the endogenous carnosine-carnosinase system in a diabetic setting, is discussed in chapter 1 from the general discussion. The second one, investigating the effect of artificially increasing plasma or tissue carnosine, will be handled in this chapter.

In **study 3**, the role of carnosine was investigated by raising plasma and muscle carnosine (through carnosine supplementation) or by raising muscle carnosine alone (through beta-alanine supplementation) in a very mild high-fat diet rat model. Our design was innovative as it allowed us to assess carnosine's site of action (plasma or tissue/muscle). In addition, using a more real-life model (high-fat diet), in contrast to the severe animal models used in the previous studies, made it possible to look at the effects of carnosine in the initial stage of metabolic disturbance. We could conclude that plasma carnosine, and not muscle carnosine, is involved in preventing early-stage metabolic stress in circulation and muscle. More specifically, carnosine supplementation (increasing both plasma and muscle carnosine) could reduce elevated plasma and urine CML (induced by high-fat diet) with ~50% and muscle iNOS mRNA with 47%, whereas beta-alanine supplementation (only increasing muscle carnosine) had no effects. The fact that only carnosine supplementation, and not beta-alanine, protected skeletal muscle from increased inflammatory signaling (although both groups equally increased muscle carnosine above normal levels), likely points to a systemic effect with skeletal muscle as a target organ.

In figure 9, several possible pathways are represented by which systemic disturbances (diet-induced) can provoke metabolic changes in skeletal muscle. Study 3 demonstrated that elevated plasma carnosine attenuates inflammatory signaling in skeletal muscle, possibly through the AGE-RAGE pathway (marked by bold lines in figure 9). *Plasma CML* is one of the most important ligands for RAGE and this RAGE-AGE interaction triggers intracellular events increasing nuclear transcription factors such as nuclear factor- kappa B (NF-κB) (Kislinger 1999). The latter can increase the transcription of inflammatory products such as TNFα and iNOS (Kislinger 1999; Adams 2002), which is known to inhibit

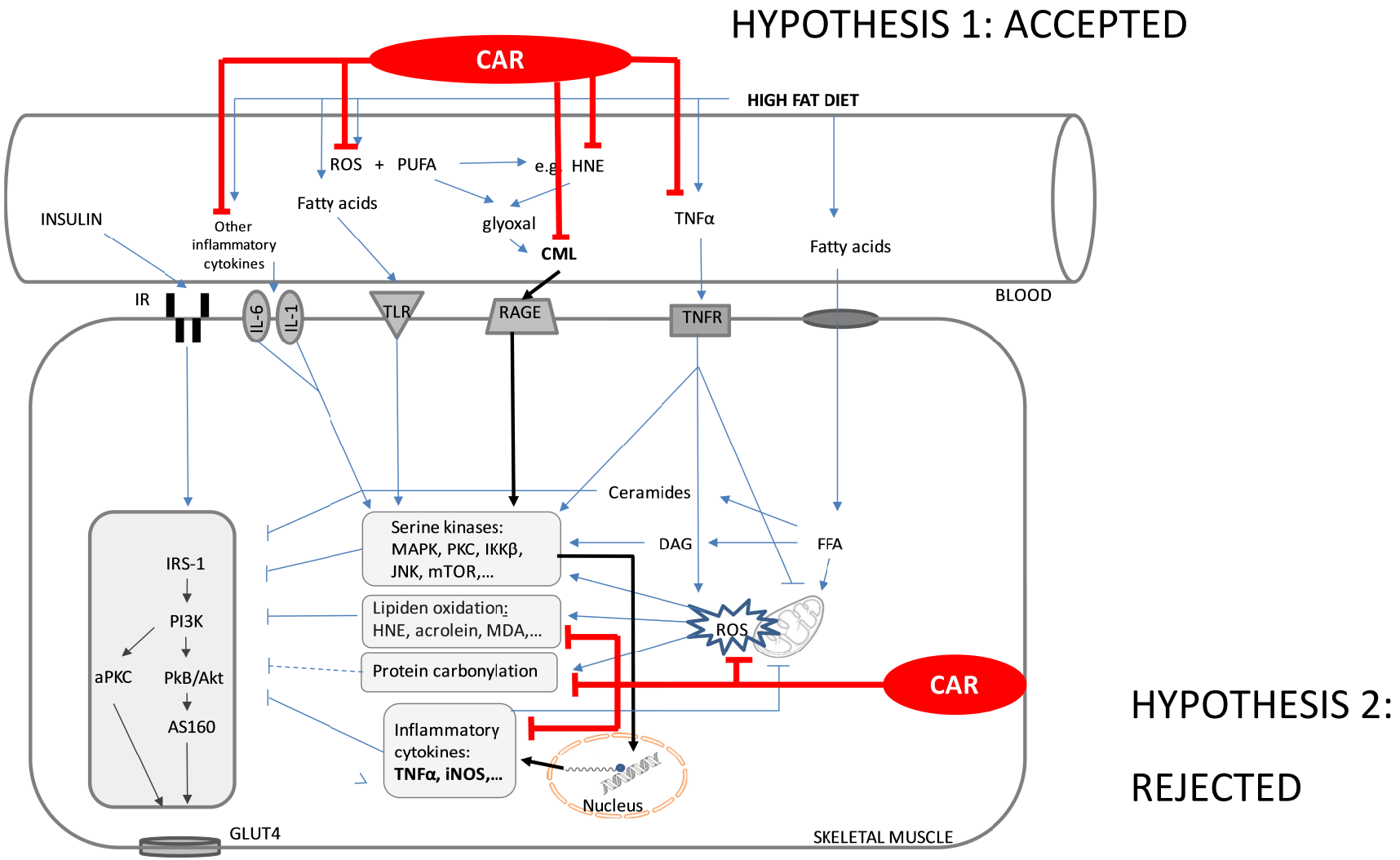


Figure 9: The most dominant pathways for high-fat diet induced metabolic stress towards skeletal muscle are presented. Study 3 of this work concluded that plasma carnosine (hypothesis 1), but not muscle carnosine (hypothesis 2), prevents high-fat diet induced metabolic stress in circulation and skeletal muscle. The pathway in the middle (black bold arrows) and glucose transport in skeletal muscle were investigated. → Stimulation, ⊣ inhibition. *Abbreviations*: ROS: reactive oxygen species, HNE: 4-hydroxy-2-nonenal, CML: carboxymethyllysine, TNFα: tumor necrose factor α, iNOS: inducible nitric oxide synthases, FFA: free fatty acids, PUFA: poly-unsaturated fatty acids, DAG: diacylglycerol, TLR: Toll-like receptor 4, RAGE: receptor for advanced glycation end-products, MDA: malondialdehyde, CAR: carnosine, IRS-1: insulin receptor substrate 1, GLUT-4: glucose transporter 4.

the insulin signaling pathway and thus insulin-induced glucose uptake in skeletal muscle (Uysal 1997; Perreault 2001; Yasukawa 2005; Plomgaard 2005; Carvalho-Filho 2006; Ropelle 2013). Hofmann et al. (2002) showed that reduced AGE intake leads to lower levels of circulating AGEs and to improved insulin sensitivity in db/db mice (Hofmann 2002). The other way around, Cassese et al. (2008) demonstrated reduced insulin-induced glucose uptake and protein kinase B phosphorylation in skeletal muscle of C57/BL6 mice receiving a high AGEs diet (Cassese 2008).

However, as presented in figure 9, this AGE-RAGE pathway is only a part of a more complex entity concerning skeletal muscle insulin resistance (Wei 2008; Abdul-Ghani 2010; Kewalramani 2010). Indeed, chronic elevated circulating fatty acids can directly negatively affect metabolic pathways in skeletal muscle (figure 9). Fatty acids can enter the muscle fiber and create (in pathological circumstances) an overproduction of diacylglycerol (DAG) or ceramides. These metabolites are known to inhibit the insulin pathway. In addition, saturated fatty acids from the circulation can activate the toll-like receptor (TLR), which can activate inflammatory pathways (Tsukumo 2007; Martins 2012).

Unfortunately, the 60% high-fat diet (study 3) was not drastic enough to significantly alter glucose tolerance (IVGTT) and glucose transport in skeletal muscle, therefore lacking a true end point of our observations. However, we observed that fasting plasma insulin was reduced by ~45% in the carnosine supplemented high-fat group compared to the high-fat group without supplementation, suggesting a better basal whole body insulin sensitivity.

Does it mean that carnosine in *skeletal muscle* has no protective function? Probably, carnosine exerts its functions in muscle as well. It is possible that increasing muscle carnosine by beta-alanine supplementation has some protective effects on insulin signaling by quenching HNE, decreasing intramyocellular CML, increasing anti-oxidant capacity,… but we assume that additionally increasing plasma carnosine provides extra benefits by acting on two sites (systemic and local effects).

Conclusion: Plasma carnosine and not muscle carnosine is involved in preventing early-stage lipid-oxidation in circulation and inflammatory signaling in skeletal muscle.

3.2. Properties of the hydrolysis products of carnosine

From the literature above, it can be concluded that the molecule carnosine carries many biochemical and physiological properties. However, carnosine is almost immediately hydrolyzed by CN1 into its two constituent amino acids beta-alanine and histidine, at least in humans. Therefore, it can be questioned whether the hydrolysis products of carnosine (beta-alanine and histidine) are accounted for some of the observed properties. Although rats (and mice) do not have CN1, Aldini et al. (2011) have demonstrated that carnosine is cleared from the blood 2 hours after carnosine supplementation (100 mg/kg body weight). In those species, the catalytical activity of carnosine mainly takes place in the kidney because of the presence of tissue carnosinase.

Table 1 provides an overview of the biochemical properties of carnosine and the hydrolysis products beta-alanine and histidine. From this table, it can be deducted that most of the functions of carnosine are related to histidine or its imidazole moiety, except for the buffering capacity and the quenching of reactive aldehydes, these properties are much better accomplished by the dipeptide. Caution is warranted however, as the references from table 1 contain *in vitro* or animal experiments.

Property	**Carnosine**	**Beta-alanine**	**Histidine**	**References**
Anti-oxidant (scavenging effect)				
Peroxyl radicals	**	/	**	(Kohen 1988; Decker 2000)
Hydroxyl radicals	**	/	**	(Boldyrev 1992; Tamba 1999)
Metal ion chelating				
Cu^{2+} chelation	**	ND	***	(Velez 2008)
Buffering activity (pKa)	**	ND	*	(Abe 2000)
Scavenging of reactive carbonyl species	**	/	*	(Aldini 2002b)
Anti-inflammatory effect	**	ND	**	(Lee 2005; Liu 2008)
Lipid lowering effect	**	ND	**	(Mong 2011)

Table 1. Comparison of the biochemical and physiological properties of carnosine with its hydrolysis products beta-alanine and histidine (based on Boldyrev 2013). ** represent equal activity as carnosine, * lower activity, *** higher activity, / no activity, ND not determined.

A side note must be made for beta-alanine. We know that the majority of ingested beta-alanine (> 90%) is metabolized in the body (meaning: not accumulated in tissue carnosine or excreted in urine). The initial step of beta-alanine catabolism is thought to be the transamination to form malonic acid semialdehyde, which can then be converted into acetyl CoA (Griffith 1986). The later has a pivotal role in the citric acid cycle, which is a series of chemical reactions to generate energy through the oxidation of acetyl CoA derived from carbohydrates, fats and proteins into carbon dioxide and chemical energy in the form of adenosine triphosphate (ATP). It can be questioned whether the contribution of acetyl CoA derived from beta-alanine is substantial enough to influence glucose and fatty acid oxidation.

4. FUTURE DIRECTIONS

Four years of swimming in carnosine's literature and performing animal and human experiments has contributed to new insights into the carnosine-carnosinase system in diabetic circumstances (cfr supra). Although the most important research questions are adequately addressed, some observations must be further clarified:

- The finding that carnosine has a systemic effect, rather than a local effect, creates an obstacle in translating the animal findings to diabetic patients and in developing a carnosine-related treatment for diabetic patients, as the highly active CN1 (not present in rodents) almost immediately hydrolyses carnosine in plasma. This has opened two research directions:

  First, it becomes interesting to compare diabetic individuals with low versus high CN1 levels for markers of metabolic stress (as we did in this work). We cautiously proposed that CN1 is associated with markers of dyslipidemia. Further research must however clarify this relationship by taking into account hypoglycemic and lipid-lowering medication.

  Second, these data have drawn the attention towards the development of carnosine derivatives being resistant to CN1 (Vistoli 2012) or towards naturally occurring carnosine related compounds with similar characteristics as carnosine (and 'more' resistant towards CN1). The fact that T2D patients display lower CN1 activity levels, compared to healthy control subjects, makes them an even more interesting population for possible carnosine-related treatments.

- Although this work disadvises treatment of diabetic patients with beta-alanine, two points of interest are necessary to make: 1) it must be determined whether *severe* diabetic patients display increased levels of muscle carnosine as well; 2) we cannot deny recent reports of decreased renal and retinal carnosine levels in animals with respectively diabetic retino- and nephropathy. Therefore, future research can focus on the potential of beta-alanine supplementation to increase carnosine levels in kidney and retina and whether these increased carnosine levels can protect the tissues from damaging species.

- We concluded in study 1 that more than 90% of ingested beta-alanine has an unknown metabolic fate. In this work we already discussed the possible role of beta-alanine transaminase (ABAT) and malonic deminaldehyde dehydrogenase (MSAD) in the degradation of beta-alanine in tissues. Understanding the unknown metabolic fate of ingested beta-alanine could lead to much more efficient muscle carnosine loading strategies.

- Last but not least, it must be clarified whether or not carnosine adducts with reactive aldehydes are detected by ^{1}H-MRS and by HPLC, in order to correctly interpret carnosine concentrations in tissues.

We can conclude that the journey does not end here and that this work has opened new perspectives towards the future.

5. CONCLUSIONS

As the main result, this work delivered new insights into 1) the endogenous carnosine-carnosinase system in a diabetic state and 2) the protective role of elevated plasma and/or tissue carnosine levels. As a by-product, some interesting elements for the sports field are provided as well. The main conclusions are presented below:

1. **The endogenous carnosine-carnosinase system is altered by a diabetic state.**

   - We demonstrated that muscle carnosine is increased in obese/diabetic circumstances, both in rodents and humans (**study 4**). However, caution is warranted as not all our high-fat diet rat models showed elevated muscle carnosine levels and as Gualano et al. (2010) demonstrated a decrease in diabetic type 2 patients. Therefore, we have put forward the hypothesis that muscle carnosine in a diabetic state is a balance between diabetes-related upregulating and downregulating factors, and depending on the factors dominating in a specific situation, muscle carnosine will be increased, decreased or remain equal.

   - We provided evidence that diabetic patients are not characterized by elevated CN1 levels. While CN1 content was equal between healthy subjects and type 2 diabetic patients, CN1 activity was even lower in the patients. Moreover, there was no relationship between CN1 (content and activity) and HbA1c (average level of blood glucose over the previous 3 months) in diabetic patients. In addition, CN1 levels are remarkably stable over a 6-month period and not influenced by chronic exercise training in a diabetic population.

2. **Increasing plasma carnosine levels by carnosine supplementation attenuates early-stage lipid-oxidation in circulation and inflammatory signaling in skeletal muscle.**

   In **study 3**, plasma and muscle carnosine were independently increased by respectively carnosine and beta-alanine supplementation to elucidate its effect on high-fat diet induced metabolic stress in circulation and tissue (muscle) in a high-fat diet rodent model. Elevated plasma carnosine levels, and not elevated muscle carnosine levels, demonstrated protective effects in both circulation and skeletal

muscle tissue, pointing towards a systemic effect with skeletal muscle as a target organ.

Taking together point 1 and 2, we can disadvise beta-alanine supplementation in diabetic patients (as suggested from literature), because 1) diabetic patients have no depleted muscle carnosine levels and 2) artificially increasing muscle carnosine levels does not attenuate metabolic stress in both circulation and skeletal muscle.

3. **This work provided an explanatory model for muscle carnosine turnover in four different phases: muscle carnosine at baseline, during loading, washout and maintenance.**

   - At baseline, muscle carnosine synthesis and degradation are in a steady equilibrium and predominantly determined by skeletal muscle fiber type, age and sex. As demonstrated above, a diabetic state can alter this steady equilibrium.

   - During beta-alanine supplementation (loading), carnosine synthesis will be elevated because of increased precursor availability and thus net turnover will be strongly positive. **Studies 1 and 2** searched for determinants of muscle carnosine loading. Only one significant determinant was found, i.e. meal-timing. Taking beta-alanine at the start of the meal results in a higher absolute (0.58 mM) and relative (23%) increase in soleus muscle compared to taking beta-alanine in between the meals, suggesting that insulin plays a role during loading. On the other hand, muscle fiber type, gender, supplementation form (slow-release vs powder beta-alanine), body weight, fat-free mass and baseline carnosine content did not influence the absolute increase in muscle carnosine during loading.

     From **study 1**, we could calculate that of the total amount of chronically ingested beta-alanine only a small portion is incorporated into muscle carnosine (2.8%) and only very little is excreted in urine (1.6%), raising a new research question: *Where does all the beta-alanine go?*

   - Upon discontinuation of chronic beta-alanine intake, a net negative balance of carnosine turnover is present, demonstrated by a slow wash-out of 6-20 weeks. Therefore, **study 2** established a suitable maintenance dose of ~1.2g beta-

alanine/day to maintain moderately elevated carnosine stores. This has useful implications for athletes and resulted in the advise to phase their beta-alanine intake towards the competition.

To summarize, this thesis provided evidence that the endogenous carnosine-carnosinase system is influenced by obesity or diabetes type 2. In contrast to what is currently believed, we demonstrated that muscle carnosine is higher and CN1 activity is lower in diabetic patients compared to healthy control subjects. Thus, alterations in the endogenous carnosine-carnosinase system in a diabetic state are rather protective and not contributing to a negative viscious circle. In addition, chronic carnosine supplementation can attenuate metabolic stress in circulation and skeletal muscle in rats.

SUMMARY - SAMENVATTING

SUMMARY OF THE THESIS

Background

It is suggested that the molecule carnosine (beta-alanyl-L-histidine) is a potential candidate to counteract the development of diabetes or diabetes-related diseases because of its protective biochemical properties. Carnosine is a naturally occurring dipeptide that is predominantly and abundantly synthesized (and stored) in skeletal muscle by carnosine synthase from its two constituent amino acids beta-alanine and L-histidine, whereas its breakdown occurs predominantly in the circulation by the highly active serum carnosinase (CN1). As carnosine is present in skeletal muscles of many vertebrates, it is consumed through the intake of (white) meat and fish.

This carnosine-carnosinase system has been associated with diabetes or diabetes-related diseases by two different research approaches. First, several genome-wide linkage scans for diabetic nephropathy have demonstrated that a polymorphism of the serum carnosinase gene (CNDP1), leading to low CN1 activity, is associated with reduced risk of diabetic nephropathy. Second, the substrates of CN1, carnosine and anserine (methylated form of carnosine), have several biochemical properties that appear to be relevant in diabetes or related diseases. In vitro studies have demonstrated that carnosine and anserine have the ability to act as an anti-oxidant, anti-glycator, metal-ion chelator and quencher of reactive carbonyls species and protein carbonyls. This would lead to suppressed formation of advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs), which are predominantly present in diabetes. Moreover, this quenching/scavenging of deleterious species has created the theory that carnosine sacrifices itself and becomes depleted in diabetic tissues.

However, despite the apparent relevance of this carnosine-carnosinase system in diabetes or diabetes-related diseases, little is known about the endogenous carnosine and carnosinase concentrations in obesity and diabetes and the protective effect of carnosine supplementation.

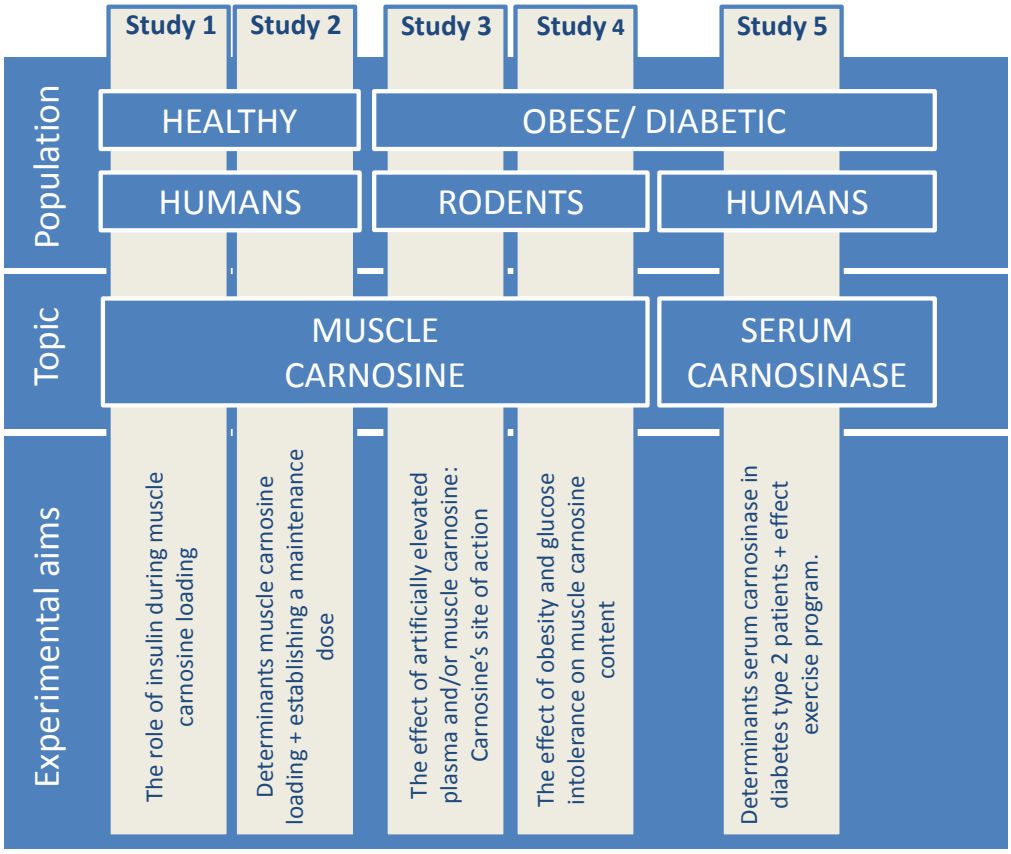
Aims of this thesis

The two main aims of this work are to provide insight into the endogenous carnosine-carnosinase system in a diabetic state (**study 4** and **5**) and to investigate whether or not elevating plasma and/or tissue carnosine levels have beneficial outcomes (**study 3**).

In the build-up to our main aims, this work also conducted two interventional studies in healthy subjects and investigated the determinants of muscle carnosine loading under normal physiological conditions (**study 1** and **2**), with the focus on insulin (key hormone in diabetes).

Research and conclusions

The figure below presents schematically the five studies in this thesis.



Conclusion 1: Muscle carnosine content and serum carnosinase are altered in diabetic circumstances.

Research: **Study 4** investigated the effect of various degrees of high-fat diets in rodents (rats and mice), provoking various degrees of obesity and glucose intolerance, on muscle carnosine concentrations. We demonstrated that muscle carnosine increases with progressive glucose intolerance. Additional human data (general discussion) could confirm this finding. However, as this is in contrast to what is currently believed, we have put forward a working model that muscle carnosine in a diabetic state is a balance between diabetes-related factors possibly increasing or decreasing baseline muscle carnosine.

Study 5 investigated CN1 content and activity in 243 diabetic type 2 patients (men and women) before and after a 6-month exercise program. Two independent observations suggest that CN1 is not increased in diabetic individuals. First, there is no correlation between HbA1c (average level of blood glucose over the previous 3 months) and CN1 content and activity in this cohort of type 2 diabetic patients. Second, while CN1 content is equal between healthy subjects and T2D patients, CN1 activity is even lower in T2D patients. Concomitantly, we found that CN1 levels are remarkably stable over a 6-month period and not influenced by chronic exercise training in a diabetic population.

Conclusion 2: Increased plasma carnosine levels, but not muscle carnosine levels, attenuates early-stage metabolic stress in circulation and skeletal muscle.

Research: In **study 3**, plasma and muscle carnosine were independently increased by respectively carnosine and beta-alanine supplementation to elucidate its effect on metabolic stress in circulation and tissue (muscle) in a high-fat diet rodent model. Elevated plasma carnosine levels, and not elevated muscle carnosine levels, resulted in protective effects in both circulation and skeletal muscle tissue, pointing towards a systemic effect with skeletal muscle as a target organ.

Conclusion 3: This work provided an explanatory model for muscle carnosine turnover in four different phases: muscle carnosine at baseline, during loading, washout and maintenance.

At baseline, muscle carnosine synthesis and degradation are in a steady equilibrium and are predominantly determined by skeletal muscle fiber type, age and sex. As demonstrated above, a diabetic state can alter this steady equilibrium.

During beta-alanine supplementation (loading), carnosine synthesis will be elevated because of increased precursor availability and thus net turnover will be strongly positive. **Studies 1** and **2** searched for determinants of muscle carnosine loading. Only 1 significant determinant was found, i.e. meal-timing. Taking beta-alanine at the start of the meal results in a higher absolute (+0.58 mM) and relative (+23%) increase compared to taking beta-alanine in between the meals in soleus muscle, suggesting that insulin plays a role during loading. On the other hand, muscle fiber type, gender, supplementation form (slow-release vs powder beta-alanine), body weight, fat-free mass and baseline carnosine content did not (or very little) influenced the absolute increase in muscle carnosine during loading.

From **study 1**, we could calculate that of the total amount of chronically ingested beta-alanine only a small portion is incorporated into muscle carnosine (~3%) and only very little is excreted in urine (~1.6%), raising a new research question: Where does all the beta-alanine go?

Upon discontinuation of beta-alanine supplementation, a net negative balance of carnosine turnover is present, demonstrated by a slow wash-out of 6-20 weeks. Therefore, **study 2** established a suitable maintenance dose of ~1.2g beta-alanine/day to maintain moderately elevated carnosine stores. This has useful implications for athletes and resulted in the advice to phase their beta-alanine intake towards the competition.

To summarize, this thesis provides evidence that the endogenous carnosine-carnosinase system is partly influenced by obesity or diabetes type 2. In contrast to what is currently believed, we demonstrated that muscle carnosine increases with progressive glucose

intolerance, both in rodents and humans. In addition, CN1 content is equal between healthy subjects and type 2 diabetes patients, but CN1 activity is lower in the patients (humans). Notwithstanding the fact that the endogenous system is actually functioning rather good, elevating plasma carnosine (and not muscle carnosine) can attenuate metabolic stress in circulation and skeletal muscle (high-fat diet rat model). The finding that carnosine has a systemic effect, rather than a local effect, creates an obstacle in the road to developing a carnosine-related treatment for diabetic patients, as the highly active enzyme serum carnosinase almost immediately hydrolyses carnosine in plasma. This opens new research perspectives towards the search for carnosine related compounds being resistant for serum carnosinase.

SAMENVATTING VAN DE THESIS

Achtergrond

De molecule carnosine (beta-alanyl-L-histidine) heeft het potentieel om de ontwikkeling van diabetes of diabetes gerelateerde ziekten tegen te gaan omwille van zijn beschermende biochemische eigenschappen. Carnosine is een dipeptide en wordt voornamelijk in grote hoeveelheden gesynthetiseerd in de skeletspier door het enzyme carnosine synthase, vertrekkende vanuit zijn twee bouwstenen (aminozuren) beta-alanine en L-histidine. Omgekeerd vindt de afbraak tot zijn twee bouwstenen voornamelijk plaats in de circulatie door het enzym serum carnosinase (CN1). Consumptie van carnosine gebeurt door het eten van (wit) vlees en vis, aangezien carnosine talrijk aanwezig is in skeletspieren van gewervelden.

Het carnosine-carnosinase systeem is recent door twee onafhankelijke onderzoeksbenaderingen geassocieerd met diabetes of diabetes gerelateerde ziekten. Ten eerste, genoomstudies voor diabetische nefropathie hebben aangetoond dat een polymorfisme van het serum carnosinase gen (CNDP1), dat leidt tot een lagere activiteit van CN1, geassocieerd is met een lager risico op diabetische nefropathie. Ten tweede, de substraten van CN1, met name carnosine en anserine (de gemethyleerde vorm van carnosine), hebben verschillende biochemische eigenschappen die beschermend zouden zijn bij suikerziekte. Carnosine heeft bijvoorbeeld anti-oxiderende en anti-glycerende eigenschappen en kan reactieve carbonyl groepen of eiwit-carbonyls vangen (en dus onschadelijk maken). Deze eigenschappen zouden aan de basis liggen van verminderde productie van schadelijke eindproducten van het suiker- en vetmetabolisme (AGEs en ALEs genaamd), die talrijk aanwezig zijn bij suikerziekte (diabetes mellitus). Daarenboven heeft dit ‘vangen’ van schadelijke moleculen de theorie doen ontstaan dat carnosine waarschijnlijk is gedaald in weefsels die onder stress staan, zoals bij diabetes.

Vanuit de literatuur wordt er dus gesuggereerd dat dit carnosine-carnosinase systeem een belangrijke rol speelt in diabetes of diabetes gerelateerde ziekten. Echter, het is niet gekend of de endogene productie van carnosine of carnosinase beïnvloed wordt door obesitas of diabetes. Daarenboven zijn er weinig in vivo studies beschikbaar over de effecten van carnosine suppletie bij deze pathologie.

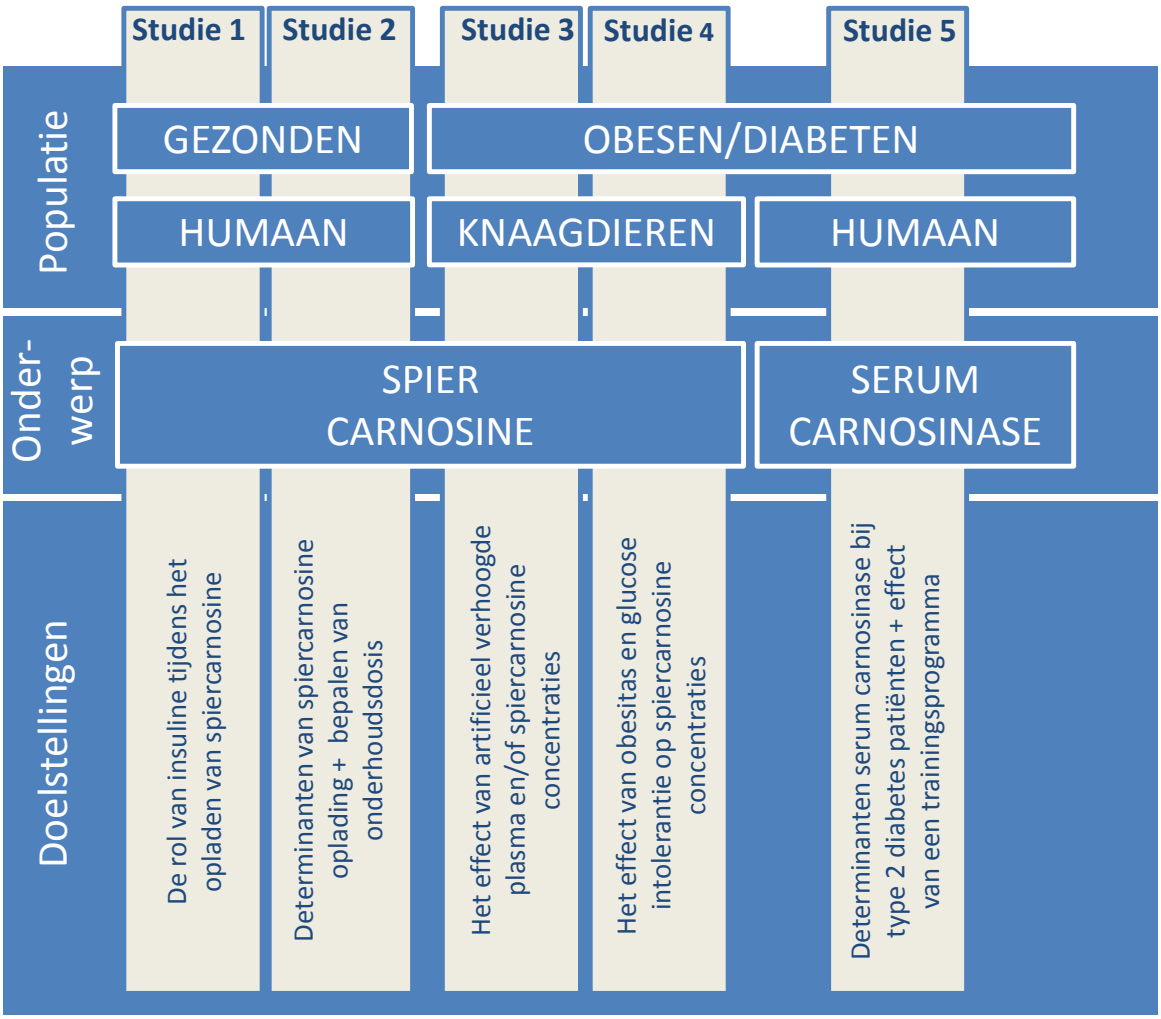
Doelstellingen

De twee voornaamste doelstellingen van dit werk zijn: 1) inzicht verschaffen over het endogene carnosine-carnosinase systeem bij obesitas en diabetes en 2) de effecten onderzoeken van het artificieel verhogen van plasma- en spier carnosine op de ontwikkeling van obesitas-geïnduceerde metabole stress.

Naar de aanloop van onze hoofddoelstellingen, zal deze thesis ook twee interventiestudies uitvoeren bij gezonde proefpersonen om de determinanten te onderzoeken van spier carnosine oplading onder normale fysiologische omstandigheden. Hierbij zal voornamelijk aandacht besteed worden aan de rol van insuline (sleutel hormoon in diabetes).

Onderzoek en conclusies

Onderstaande figuur geeft schematisch de vijf studies weer uit deze thesis.



Conclusie 1: Spier carnosine en serum carnosinase concentraties worden beïnvloed door glucose intolerantie en diabetes.

Onderzoek: **Studie 4** heeft het effect onderzocht van verschillende vet diëten, die tot doel hadden om verschillende gradaties in obesitas en glucose-intolerantie te veroorzaken, op spier carnosine concentraties bij knaagdieren (ratten en muizen). Spier carnosine concentraties bleken te stijgen naargelang de glucose intolerantie toenam. Daarenboven kon additionele humane data deze bevinding bevestigen. Echter, dit is in tegenstelling met de theorie dat spier carnosine zichzelf opoffert om bijvoorbeeld reactieve carbonyl groepen onschadelijk te maken, waardoor verondersteld werd dat carnosine concentraties zouden dalen. Daarom heeft deze thesis een werkmodel naar voor gebracht met verschillende diabetes-gerelateerde factoren die spier carnosine concentraties zouden kunnen doen stijgen of dalen.

Studie 5 heeft CN1 concentratie en activiteit onderzocht in 243 patiënten met diabetes type 2 (mannen en vrouwen) voor en na een 6-maand durend bewegingsprogramma. Twee onafhankelijke observaties tonen aan dat CN1 niet gestegen is bij diabeten. Ten eerste, er is geen correlatie tussen HbA1c (bloedsuiker over de voorbije 3 maanden) en CN1 concentratie en activiteit in de type 2 diabetes patiënten. Ten tweede, CN1 content is gelijk tussen diabeten en gezonden en CN1 activiteit was zelfs significant lager in de patiënten populatie. Daarnaast hebben we kunnen aantonen dat CN1 zeer stabiel is over de tijd (6 maanden) en dat het niet beïnvloedbaar is door een bewegingsprogramma.

Conclusie 2: Het verhogen van plasma carnosine, en niet het verhogen van spier carnosine, onderdrukt vroegtijdige metabole stress in de circulatie en in de skeletspier bij knaagdieren.

Onderzoek: In **studie 3** werd het effect onderzocht van verhoogde plasma- en spier carnosine concentraties, door respectievelijk carnosine en beta-alanine supplementatie, op metabole stress in de circulatie en weefsels in hoog-vet dieet-gevoede ratten. De studie kon aantonen dat enkel het verhogen van plasma carnosine bescherming bood in de circulatie en de weefsels, wat duidde op een systemisch effect met de spier als doelorgaan.

Conclusie 3: Dit werk heeft een verklarend model aangeboden voor spier carnosine turnover (netto verandering) in vier verschillende fases: spier carnosine op basis niveau, tijdens oplading, tijdens het uitwassen naar basis niveau en tijdens het onderhouden van verhoogde concentraties.

Op basis niveau zijn spier carnosine synthese en afbraak in evenwicht met elkaar en wordt het voornamelijk bepaald door spiervezeltypering, leeftijd en geslacht. Hierbij hebben we aangetoond dat diabetes dit evenwicht kan verstoren.

Tijdens beta-alanine suplementatie (oplading), zal carnosine synthese verhogen door gestegen precursor beschikbaarheid waardoor de turnover sterk positief zal zijn. **Studie 1** en **2** zochten naar determinanten van spier carnosine oplading. Slechts 1 determinant werd gevonden, namelijk het gelijktijdig innemen van beta-alanine met de maaltijden. Wanneer beta-alanine aan de start van de maaltijd werd genomen, zag men een hogere absolute (+0.58 mM) en relatieve (+23%) toename in de soleus spier, waardoor gesuggereerd werd dat insuline een rol speelt tijdens oplading. De andere onderzochte determinanten (spiervezeltype, geslacht, supplementatie vorm ‘snel versus traag werkende’, lichaamsgewicht, vet-vrije massa en spier carnosine bij aanvang) hadden geen (of zeer weinig) invloed op de absolute stijging in spier carnosine. Ten slotte hebben we aangetoond dat slechts ~3% van de ingenomen beta-alanine wordt opgenomen in de spier en ook zeer weinig (~1.6%) wordt uitgescheiden in de urine, waardoor een nieuwe onderzoeksvraag is ontstaan: ‘Waar gaat het merendeel van de ingenomen beta-alanine naar toe in ons lichaam?’

Nadat dagelijkse beta-alanine inname wordt stopgezet, wordt de balans van carnosine turnover negatief, dat is aangetoond door het zeer traag uitwassen van de spier (6-20 weken). Daarom heeft **studie 2** een geschikte onderhoudsdosis voorgesteld van ~1.2g beta-alanine/dag om verhoogde spier carnosine niveaus te verzekeren. Deze bevinding is van belang voor atleten waardoor we nu het advies uitschrijven om de beta-alanine supplementatie op te delen in fases met een piek naar de competitie periodes.

We kunnen dus **samenvatten** dat deze thesis bewijs levert dat het endogeen carnosine-carnosinase systeem in beperkte mate wordt beïnvloed door obesitas en diabetes type 2. In tegenstelling tot wat momenteel wordt verondersteld, hebben we aangetoond dat spier carnosine stijgt met toenemende mate van glucose intolerantie (zowel dierexperimenteel als humaan). Daarnaast blijkt CN1 content niet te veranderen door diabetes en CN1 activiteit was in onze studie zelfs lager bij de diabeten dan bij de gezonden. Niettegenstaande dat het endogene systeem vrij goed functioneert, helpt het verhogen van plasma carnosine (en niet het verhogen van spier carnosine) tegen metabole stress in de circulatie en het spierweefsel (bij ratten die een vet-dieet hebben gevolgd). De bevinding dat carnosine een systemisch effect heeft, en geen lokaal effect, vormt een probleem om deze toepassing te vertalen naar mensen, aangezien zij het zeer actieve serum carnosinase enzym bezitten. Dit opent de zoektocht naar carnosine-achtige stoffen die resistent zijn aan het enzym en we kunnen stellen dat er zich reeds interessante kandidaten hebben aangeboden.

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REFERENCE LIST

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PERSONAL LIST OF PUBLICATIONS

A1

Stegen S, Sigal R, Kenny G, Kwandwala F, De Heer E, Yard B, Calders P, Peersman W, Derave. Serum carnosinase content and activity in type 2 diabetic patients. *(manuscript in progress)*

Stegen S, Everaert I, Deldique L, Hespel P, Derave W. Increased muscle histidine-containing dipeptides in glucose intolerant rodents. *(manuscript in progress)*

Stegen S, Stegen B, Aldini G, Altomare A, Cannizzaro L, Orioli M, Gerlo S, Deldique L, Ramaekers M, Hespel P, Derave W. Plasma carnosine, but not muscle carnosine, attenuates high-fat diet induced metabolic stress. *Amino Acids* (submitted).

Stegen S, Bex T, Vervaet C, Vanhee L, Achten E and Derave W. The Beta-Alanine Dose for Maintaining Moderately Elevated Muscle Carnosine Levels. *Med Sci Sports Exerc* 2014 (Epub ahead).

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

Bex T, Chung W, Baguet A, **Stegen S**, Stautemas J, Achten E and Derave W. Muscle carnosine loading by beta-alanine supplementation is more pronounced in trained vs. untrained muscles. *J Appl Physiol* (1985) 116: 204-209, 2013.

Everaert I, **Stegen S**, Vanheel B, Taes Y and Derave W. Effect of Beta-Alanine and Carnosine Supplementation on Muscle Contractility in Mice. *Med Sci Sports Exerc* 45: 43-51, 2013.

Gualano B, Everaert I, **Stegen S**, Artioli GG, Taes Y, Roschel H, Achten E, Otaduy MC, Lancha-Junior AH, Harris R and Derave W. Reduced muscle carnosine content in type 2, but not in type 1 diabetic patients. *Amino Acids* 43: 21-24, 2012.

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

D'hooge R, Hellinckx T, Van Laethem C, **Stegen S**, De SJ, Van AS, Dewolf D and Calders P. Influence of combined aerobic and resistance training on metabolic control, cardiovascular fitness and quality of life in adolescents with type 1 diabetes: a randomized controlled trial. *Clin Rehabil* 25: 349-359, 2011.

Elmahgoub SS, Calders P, Lambers S, **Stegen S**, Van Laethem C and Cambier DC. The effect of combined exercise training in adolescents who are overweight or obese with intellectual disability: the role of training frequency. *J Strength Cond Res* 25: 2274-2282, 2011.

Stegen S, Derave W, Calders P, Van Laethem C and Pattyn P. Physical fitness in morbidly obese patients: effect of gastric bypass surgery and exercise training. *Obes Surg* 21: 61-70, 2011.

Elmahgoub SM, Lambers S, **Stegen S**, Van Laethem C, Cambier D and Calders P. The influence of combined exercise training on indices of obesity, physical fitness and lipid profile in overweight and obese adolescents with mental retardation. *Eur J Pediatr* 168: 1327-1333, 2009.

A3

Everaert I, **Stegen S**, Vanheel B, Taes Y, Derave W. Nutritional manipulation of histidine-containing dipeptides in mouse skeletal muscle: effects on contractility. *Sport & Geneeskunde*

Stegen S, Everaert I, Derave W. Het gebruik van voedingsupplementen bij jongeren. *Tijdschrift voor lichamelijke opvoeding* 2, 19-21

C1-C3

6th Congress of the International Federation for the Surgery of Obesity and Metabolic Disorders – Brussels, Belgium – 1-3 May, 2014

Stegen S, Calders P, Derave W, Pattyn P. Importance of exercise after bariatric surgery.
(oral presentation)

18th annual European College of Sport Sciences Congress - Barcelona, Spain – 26-29 June, 2013

Stegen S, Blancquaert L, 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)

17th *Symposium of the Flemish Society for Kinesiology – Brussels, Belgium -7 December, 2012*

Stegen S, Blancquaert L, Everaert I, Bex T, Taes Y, Vanhee L, Vervaet C, Calders P, Achten E, Derave W. Meal en beta-alanine co-ingestion enhances muscle carnosine loading, but has no effect on whole body beta-alanine retention.
(poster)

15th International Biochemistry of Exercise Congress – Stockholm, Sweden - 17-21 June, 2012

Stegen S, Stegen B, Hespel P, Richter E, Gerlo S, Aldini G, Marica O, Derave W. The effect of chronic L-carnosine and beta-alanine supplementation on obesity induced metabolic disorders in high-fat fed rats.
(poster presentation)

2nd International congress on Carnosine in exercise and disease – Ghent, Belgium -10-12 July, 2011

Stegen S, Stegen B, Hespel P, Richter E, Derave W. The effect of chronic L-carnosine and beta-alanine supplementation on glucose tolerance in high-fat fed rats.
(poster presentation)

15th Symposium of the Flemish Society for Kinesiology – Antwerp, Belgium – 10 December, 2010

Stegen S, Pattyn P, Derave W, Calders P. Subsarcolemmal and intramyofibrillar mitochondria and lipids in skeletal muscle of morbidly obese patients: extreme weight loss and exercise.
(poster presentation)

Consensus meeting Johnson & Johnson – Brussels, Belgium - 27-29 October, 2010

Stegen S, Calders P, Derave W, Pattyn P. Exercise programs in gastric bypass patients *(invited speaker)*

14th International Biochemistry of Exercise Conference (Canada) – Guelph, Canada - 1-4 June, 2009

Stegen S, Elmahgoub SS, Lambers S, Van Laethem C, Cambier D, Calders P. The influence of combined strength and endurance training on indices of obesity, physical fitness and lipid profile in overweight and obese adolescents with mental retardation. *(Poster presentation)*

44th Congress of the European Society for Surgical Research – Nîmes, France – 20-23 May, 2009

Stegen S, Calders P, Derave W, Pattyn P. Why exercising after gastric bypass surgery? *(Oral presentation)*

6th International Obesity Surgery Expert Meeting – Saalfelden, Austria - 9-12 March, 2008

Stegen S, Calders P, Derave W, Pattyn P. Combined exercise training after bariatric surgery: Why and How? *(Oral presentation)*