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An Integrated proteomic and analytical approach for elucidating the mechanism of action of histidine dipeptides and synthetic derivatives

CHIM/08

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ABSTRACT

β-alanil-L-histidine (i.e. carnosine) is an endogenous peptide that have been extensively characterized for a number of *in vitro* properties (i.e. metal chelating, antioxidant, reactive carbonyl species quenching). Several clinical trials highlighted the potential benefits of carnosine in the treatment of oxidative stress-based diseases, although the *in vivo* mechanism of action is not known, yet. The research project herein tries to expand upon the *in vivo* mechanism of action of carnosine. New analytical methods have been developed by means of liquid chromatography – tandem mass spectrometry for the quantification of histidine dipeptides, their derivatives, and the adducts formed with reactive carbonyl species into biospecimens.

A first step was the implementation of hydrophilic interaction chromatography to skip some sample preparation steps and to reduce the chance of systematic errors. The method allowed the quantification of carnosine and carnosinol (a carnosine derivative stable to carnosinase) in biospecimens. Carnosinol tissue distribution in animal models of metabolic syndrome was determined and carnosinol-acrolein adduct was detected for the first time in liver matrices. This finding experimentally confirmed the reactive carbonyl species (RCS quenching activity of histidine dipeptides and derivatives *in vivo*. However, the metabolic instability of carnosinol-HNE adduct was proved and such an evidence requires further studies aiming at understanding the metabolic fate of RCS-adducts to characterize their disposal.

Subsequently, a new method for the measurement of carnosine hydrolysis in serum was developed as well. Human serum carnosinase has been identified as the enzyme responsible for such an activity. Compared to other published assays, the method employs a direct detection of the substrate and the use of less sample. Competition experiments with either natural derivatives or other molecules were set to identify hit compounds acting as carnosinase inhibitors. The collected data were shared with computational chemists who identified putative hit compounds via docking, virtual screening, and molecular dynamic approaches.

Furthermore, a novel carnosine mechanism of action was studied starting from the evidence that carnosine can prevent the formation of protein adducts with 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) (i.e. an aldehyde intermediate of norepinephrine metabolism). This could be relevant for the in vivo mode of action of carnosine since DOPEGAL can accumulate in cells because of oxidative stress and as it covalently binds proteins, it can alter their structures and functions.

Carnosine quenching activity via the formation of an Amadori product with DOPEGAL was determined *in vitro* and in cell lysates producing DOPEGAL from enzymatic transformation of norepinephrine. Future studies should be done to characterize the metabolic stability of the adduct and its formation in biospecimens as potential biomarker of norepinephrine toxicity.

Finally, the project included proteomics studies on human umbilical vein cells (HUVECs) to assess the impact of carnosine and carnosinol on protein expression. It is widely recognized that drugs exert their pharmacological effects also by an alteration of biological pathways by modifying protein expression. Carnosine and carnosinol have little or no impact on protein expression as detectable on proteome or secretome of healthy endothelial cells. In the future the impact on pathological cells should be carried out as well. These data support the hypothesis of a low toxicity for these molecules, making them suitable candidates for a chronic administration.

Although a lot of questions are still unanswered, these data have given new insights in the mechanism of action of carnosine and in the discovery of molecules acting either as carnosine-like compounds or as carnosinase inhibitors.

RIASSUNTO

β-alanil-L-istidina (carnosina) è un peptide endogeno che possiede innumerevoli proprietà (chelante dei metalli, antiossidante, sequestrante delle specie reattive carboniliche). Diversi studi clinici hanno dimostrato un'attività farmacologica della carnosina in malattie su base ossidative, tuttavia il meccanismo dell'attività *in vivo* non è ancora noto. Questo progetto ha come scopo quello di comprendere il meccanismo *in vivo* della carnosina. Per far ciò, sono stati sviluppati nuovi metodi analitici di cromatografia liquida accoppiata a spettrometria di massa per la quantificazione in campioni biologici dei peptidi istidinici, loro derivati e gli addotti con le specie reattive carboniliche.

Come prima cosa, un metodo analitico basato su una colonna ad interazione idrofiliche è stato sviluppato per l'analisi del carnosinolo in matrici biologiche di modelli animali di sindrome metabolica. La concentrazione di carnosinolo è stata determinata in diversi tessuti e, per la prima volta, l'addotto carnosinolo-acroleina è stato identificato in omogenato di fegato. Questo conferma l'attività del carnosinolo e dei peptidi istidinici come sequestranti delle specie reattive carboniliche *in vivo*. Tuttavia, è stata identificata l'instabilità metabolica dell'addotto carnosinolo-HNE in diversi tessuti. Saranno quindi necessari ulteriori studi per la caratterizzazione del metabolismo di questi addotti e l'identificazione della corretta entità chimica da ricercare nelle matrici biologiche come indice dell'attività sequestrante di carnosina e derivati.

Il metodo basato su colonne ad interazioni idrofiliche è stato anche utilizzato per sviluppare un metodo a rivelazione diretta per determinare l'attività idrolitica del siero umano della carnosina. La carnosinasi serica è stata identificata come principale enzima impiegato nel metabolismo della carnosina. Rispetto ad altri metodi pubblicati in letteratura, quello sviluppato in questo elaborato si basa su una determinazione diretta della carnosina, senza dover effettuare processi complessi di preparazione del campione. I dati ottenuti sono stati convalidati con dati presenti in letteratura, dimostrando che il nostro metodo risulta essere

affidabile ed accurato. È stato possibile anche condurre esperimenti di competizione fra substrati naturali e alcune molecole per valutare le principali interazioni substrato/enzima, con l'obiettivo di identificare inibitori della carnosinasi. I dati ottenuti sono stati condivisi con colleghi chimici computazionali che attraverso esperimenti di *docking*, *virtual screening* e dinamica molecolare hanno identificato dei possibili inibitori naturali della carnosinasi serica umana.

Un nuovo meccanismo d'azione della carnosina è stato approfondito, in quanto recenti pubblicazioni hanno evidenziato un ruolo della carnosina nella prevenzione della formazione di addotti fra la 3,4-diidrofenilglicolaldeide (DOPEGAL), un metabolita intermedio del catabolismo della noradrenalina, e le proteine.

La capacità della carnosina di legare covalentemente la DOPEGAL tramite la formazione di un prodotto di Amadori è stata determinata *in vitro* e in lisato cellulare dove la DOPEGAL è stata formata aggiungendo noradrenalina al lisato enzimaticamente attivo. Studi futuri dovranno caratterizzare la stabilità metabolica di quest'addotto e le caratteristiche della sua formazione in matrici biologiche in quanto risulta essere un interessante biomarcatore di tossicità noradrenalinergica.

In fine è stata valutato l'impatto della carnosina e del carnosinolo sul proteoma di cellule endoteliali umane derivanti dalla vena ombelicale. È ormai noto che i farmaci non agiscono unicamente col meccanismo d'azione per il quale sono stati sviluppati, ma possono interferire con l'espressione delle proteine cellulari, aumentandone, o diminuendone l'espressione e di conseguenza attivando o disattivando vie biologiche. Carnosina e carnosinolo non inducono una variazione nell'espressione delle proteine in cellule sane. Questo conferma la sicurezza delle molecole, soprattutto prevedendone un uso come terapia cronica. In futuro l'effetto del trattamento andrà valutato su cellule in condizioni patologiche, per comprendere se, in queste condizioni, carnosina o carnosinolo riescono a influenzare vie metaboliche e risposte cellulari.

Sebbene ci siano ancora diverse domande che sono rimaste senza risposta, i dati ottenuti in questo elaborato hanno portato all'aumento della conoscenza del meccanismo d'azione di carnosina e derivati e all'identificazione di composti inibitori della carnosinasi.

INDEX

1 Introduction

1.1 REACTIVE OXIGEN SPECIES

Oxidation is the loss of electrons by a molecule, electrons that are usually transferred to another molecule undergoing to a reduction process.

Oxidation and reduction are intimate processes that coexist and are known as Redox. Redox are involved in most of the important reactions in the living organism such as cellular respiration and most of enzymatic catalyzed reactions. Redox products are often stable compounds that can be either the final molecule or an intermediate of biochemical pathways. However, redox products can sometimes be highly reactive as in the case of radicals. These compounds have short lifetime, but they initiate a cascade of radical redox reactions with biomolecule, eventually producing toxicity and cell death [1].

Reactive Oxygen Species (ROS) are a family of highly reactive molecules normally produced as result of the oxygen metabolism in cells (e.g. superoxide ion, hydroxyl radical and peroxides) [2]. ROS are known oxidants that react with nucleophiles such as lipids, proteins, and DNA, yielding to structural modification, loss of function and cell toxicity [3]. Healthy cells have well established protecting systems to manage those highly reactive molecules such as antioxidant enzymes and molecules. Antioxidant systems can deactivate radicals, generating non-toxic byproducts and blocking further damage [2].

In particular case, cells undergo to a so-called oxidative stress, when a huge amount of ROS are generated, and antioxidants defense are not sufficient to limit damages. ROS then reacts with biomolecules inducing several levels of damages, based on the cell status. Oxidative stress has been related to many chronic neurodegenerative, cardiovascular diseases and metabolic disorders [4–6].

1.2 REACTIVE CARBONYL SPECIES

One of the main targets of ROS are PUFAs. The reaction yields the formation of toxic aldehydes such as acrolein, 4-hydroxynonenal and 4-hydroxyexenal [7]. As for ROS, these molecules are extremely reactive with nucleophiles.

However, compared to the former they have a longer half-life, making them more toxic than ROS since they can circulate in the cell or even in the tissue before reacting with a biomolecule. These lipids derivative aldehydes fall under a big class of reactive molecules called reactive carbonyl species (RCS) [7]. RCS have been studied in the past decades and are connected to the onset and progression of several pathologies such as metabolic syndrome [8], diabetes [9], neurodegenerative disorders [8], etc.

Several strategies are under investigation to develop treatment that can prevent or revert the effects of RCS. Among these, RCS sequestering agents are a promising class [10]. The goal of these molecule is to be more reactive than endogenous nucleophile (e.g. glutathione, proteins, DNA), with RCS forming stable adducts. Subsequently these adducts can be further metabolized and eventually disposed via excretion.

These sequestering agents need to be bioavailable (i.e. absorbed and not substantially metabolized), reactive against RCS, but not reacting against endogenous electrophiles such as pyridoxal (an important coenzyme of biological functions).

1.3 L-CARNOSINE

β-alanil-L-histidine (i.e. L-carnosine) (figure 1.1) is an endogenous histidine dipeptide mostly present in muscle tissues [11], it is synthetized by carnosine synthase (EC 6.3.2.11) from L-histidine and β-alanine [12]. Carnosine is one of the most studied and promising sequestering agents. It reacts with RCS such as 4-hydroxy-trans-2-nonenal (HNE), 4-hydroxy-trans-2-hexenal (HHE), Acrolein, [13] Malondialdehyde(MDA), Methylglyoxal (MGO) [14] selectively, not reacting with

endogenous aldehyde such as pyridoxal [15]. L-carnosine was tested in vitro and in vivo in several models of oxidative stress diseases providing promising results in the prevention and/or regression of such pathologies [16–18].

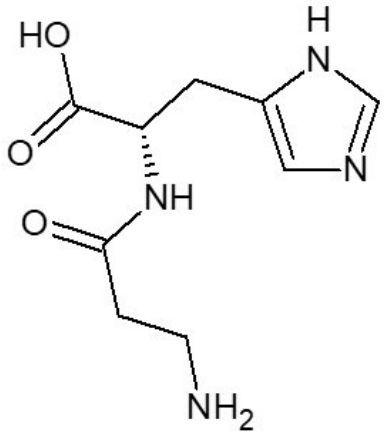

***Figure 1.1**: L-carnosine*

In humans the main drawback of a carnosine-based intervention is the low bioavailability of the peptide owing to an endogenous enzyme called human serum carnosinase (CN1 - E.C. 3.4.13.20) [19], which rapidly hydrolyzes L-carnosine upon absorption. The resulting amino acids are then distributed, and carnosine can be re-synthesized only in tissues expressing the enzyme carnosine synthase [11]. Several clinical trials were carried out in the past years, testing carnosine efficacy on several pathologies (i.e. obesity [20], cardiometabolic risk [21]). The clinical trial had positive output, nevertheless the activity of carnosine is lower than the results in animal models and the dose of carnosine necessary to reach a detectable concentration of carnosine in blood (2 g / day) is too high for a therapeutic application [20].

Several strategies have been evaluated to exploit carnosine beneficial effect. Currently the two main proposals are:

- The development of carnosine derivatives that maintain the sequestering activity but are stable to carnosinase hydrolysis (i.e. carnosinol [22]).
- The development of potent carnosinase inhibitors that can be co-administrated with carnosine to increase its bioavailability (i.e. carnostatine [23]).

Even though years of research were conducted on such an interesting molecule, several questions are still in need of an answer.

1.4 REFERENCES

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2 Aim of the Research

This work has as a main objective to understand the mechanism of action of histidine dipeptides. L-carnosine, among these, is the most known and studied [1]. Its properties in vitro are well described [2] as well as its pharmacological activities in animal models [3–5]. The scientific community thinks that carnosine activities is due to a combination of its metal chelating, antioxidant, and carbonyl scavenging properties. Nevertheless, a clear confirmation of these have not yet been published. Specifically, some of the open questions are:

- If carnosine prevents aberrant modification of proteins by sequestering reactive carbonyl species, why it is hard to detect any of these adducts in biospecimens?
- If It is hard in animal model where carnosinase is not present than it is even harder in humans. Are the above-mentioned properties the real mechanisms of action of carnosine or there is something else we are not considering yet?

The PhD project has been shaped to try answering to these questions. The following main hypotheses have been considered as research goals worth to be investigated to increase the knowledge around such an interesting molecule.

a. Does carnosine (or its derivatives) react with RCS in animal tissue?
b. Since it is hard to detect such adducts in biospecimens, are these adducts stable?
c. Does carnosine have other unveiled mechanism of actions?

These hypotheses raised analytical challenges; therefore, new analytical methods need to be developed. The current analytical methods available for carnosine analysis in biospecimens are not at the state of the art for liquid chromatography – mass spectrometry hyphenation.

For each of the research challenge in this dissertation a method is tailor-designed and applied to obtain the desired results. Methods are developed to use as small amount as possible of sample (since the low availability of biospecimens) and to be easy and fast.

Herein the following topics are discussed:

1. **Development of analytical method for the analysis of carnosine in biospecimens**: all the methods developed regarding the PhD project are here reported and described in detail. The novelty in these methods stands in the use of a "new" chromatographic separation technique and the sample preparation. Stable isotope was implemented as internal standard and a method for the chiral resolution of carnosine enantiomers was developed too.
2. **Histidine dipeptides react with RCS in animal models**: a deep investigation of the activity of carnosinol in animal model was carried out.
3. **Metabolic fate of carnosine-RCS adducts**: an extensive literature review was carried out to understand the current knowledge on carnosine-RCS adducts metabolism. Many aspects of the metabolism are still unknown, and this review put the basis for further perspective.
4. **Discovery of inhibitors of human serum carnosinase**: preliminary experiments for the discovery of hit compounds as inhibitors of serum carnosinase were carried out.
5. **Carnosine reacts with the endogenous catecholaldehyde 3,4-Dihydroxyphenylglycolaldehyde (DOPEGAL)**: deriving from MAO metabolism of norepinephrine and epinephrine, DOPEGAL has a crucial toxic role in cardiovascular diseases. For the first time activity of carnosine as quencher agent of DOPEGAL was evaluated in buffer and cell lysate.

6 Human umbilical vein cells (**HUVECs) proteomics evaluation of carnosine and carnosinol treatment**: proteomics analysis allows to evaluate whether drugs have an impact on the cellular protein expression, therefore on biochemical pathways. Via means of label free quantitative proteomics and statistical analysis the effect of carnosine and carnosinol on the expression of proteome and secretome in HUVECs was evaluated.

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3 Development of analytical method for the analysis of carnosine in biospecimens

3.1 INTRODUCTION

In the past century reverse phase chromatography have characterized most of the HPLC separation method developed [1]. Reverse phase chromatography has the advantage to exploit the hydrophobic interaction of molecule with the C_n chain (e.g. n=18) of the derivatized silica beads [2]. Ingenious alternatives have been developed for the retention of more hydrophilic molecule such as small peptides. Among all, derivatization process (e.g. FMOC) [3] or the use of ion paring agent (e.g. TFA; NFPA) [4]. Nevertheless, both these techniques have their drawbacks compared to a direct detection. Derivatization processes may not have a quantitative yield, especially if carried out in complex matrices, and have less reproducible results. Ion paring agent have beneficial effects if a UV detector is used coupled to the HPLC system. On the contrary with a mass spectrometry detector these molecules can give a phenomenon known as ion suppression [5], thus reducing the sensitivity of the measurement. Moreover, they remain stuck on metal surfaces of the instrument giving high noise level especially in negative ion mode. Starting from early 2000s a new chromatographic technique has been substantially developed and reached the market. The technique is based on liquid-liquid partitioning and hydrophilic interaction and it is called Hydrophilic Interaction Chromatography (i.e. HILIC) [6]. The elution is due to water itself or to the ion strength of the eluent that can compete with the hydrophilic interaction of the analyte with the water enriched layer residing on the surface of the stationary phase. HILIC presents several advantages when coupled to MS spectrometer: a) it uses a high percentage of organic solvents that facilitate the nebulization of the liquid in the source [7]; b) it is coupled to volatile salts that doesn't give ion suppression (e.g. ammonium formate; ammonium acetate) [8].

Histidine dipeptides are hydrophilic molecules that thorough the past years have challenged the researchers for the setup of chromatographic separation on reverse-phase chromatography.

In literature several articles are found where carnosine, or its derivatives, where analyzed after derivatization (i.e. 1-fluoro-2,4-dinitrobenzene) [9]; or with the use of ion paring agent [10]. More recently, thanks to the spreading of HILIC column, their direct chromatographic separation was achieved in methods coupled with UV detector [11] and mass spectrometry [12].

Besides the separation of the interested analytes there are different aspects that must be evaluated for the analysis in complex matrices such as biospecimens (i.e. urine, tissue homogenates and serum). These matrices are constituted by an elevated number of molecules that can compete with both: the interaction in the column or the ionization in the mass spectrometry [13]. These phenomena contribute to what is called matrix effect, i.e. the change in the response, peak shape, or analysis results when the analyte is dissolved in different matrices. Therefore, several sample preparation steps are done to reduce the number of interferences and improve the sensitivity of the method. Among all, it is to note solid phase extraction (SPE) [14] and protein precipitation [15]. Although the sensitivity of the analysis is improved the increase number of passages necessary to have the sample ready for the analysis, increase the probability to have systematic error (i.e. the human error) and so the bias of the analysis.

The ideal analytical method should have no sample preparation steps, good sensitivity, good quantification range, good precision, and accuracy. Unfortunately, this method is impossible to achieve since random and systematic errors exist. A good practice to reduce systematic error is to reduce the number of human passages at minimum. Instead for the random error the only way to control it is using an internal standard (IS).

Internal standard in liquid chromatography have different characteristics based on the detector.

For the UV detector it is suitable as IS any isomer of the analyte of interest that elute with a similar retention time and have a similar attenuation coefficient (ε). Similar retention time is needed to keep an analyte-like solvent composition to minimize solvent-dependent UV absorption changes.

Similar attenuation coefficient is needed to the IS to be affected in a similar way to the analyte concerning instrumental dependent variation of the response. In this case the ratio of analyte and Is peak areas ensure compensation for any error that may affect the analysis.

For a detector such as a mass spectrometer, the characteristics required for the IS are completely different. To note that in mass spectrometry the ionization rate of analytes is not mathematically described as is the absorbance of a chromophores (i.e. Lambert-Beer law). For instance, the ionization process in the electrospray is influenced by several conditions such as: flow instability, solvents percentage and composition, background noise, mobile phase, sample interferences and competition from bulk ion [16–18]. One of the most know interfering phenomena is the so-called ions suppression, that occurs when other less volatile molecules coelute with the analyte. The less volatile molecule can change the efficiency of the droplet formation, which affects the amount of charge ions that ultimately reach the detector [16]. This process affects the sensitivity of the instrument and the quantitative analysis on biological matrices. For this reason, the use of an internal standard as defined for the UV detector is not possible. Particularly, if the internal standard does not have the same retention time, it elutes with different solvents composition and coeluting interferents, thus leading to a different ionization yield. Moreover, similar molecules in mass spectrometry can have completely different intrinsic ionization yield. In this case one popular solution is to use the stable isotope dilution method. This method uses as IS a stable isotope of the molecule, with at least 3 m/z unit of difference with low accuracy mass spectrometer (e.g. deuterated analogue) [19].

The stable isotope analogue has the same retention time, same intrinsic ionization yield and will be affected in the same way from the interferents from the sample [20, 21].

The internal standard can reduce the effect of random and systematic error. Therefore, it is important to add it as early as possible during the sample preparation process [22].

Sometime is not possible to add it at the beginning of the sample manipulation, for example if working with tissue homogenates where the analyte is metabolized, the internal standard will be metabolized too.

Unfortunately, an unbiased analysis without error is not achievable. Random error and variation due to biological organism variability will always occur. The challenge in the development of analytical method for analyzing biospecimens is to develop a method that involve less sample manipulation as possible and reduce the addition of source of error in the instrumental analysis (e.g. direct detection preferred to derivatization procedure), giving a reliable and reproducible results.

The aim of the project was to develop direct analytical method for the analysis of histidine dipeptides and their RCS adducts in several matrices that involved less sample preparation as possible.

Carnosine is also already on the market since it is a component of food products and several food supplements. These products are subjected to quality control analysis for the release of the batch. In this contest the first method for the direct and simultaneous detection of carnosine enantiomers and natural derivative was developed. Carnosine has a stereogenic center and two configurations, where only the natural form L-carnosine is bioactive. The artificial enantiomer D-carnosine is not bioavailable [23].

3.2 Development of a direct method for the quantification of histidine dipeptides in biological matrices.

3.2.1 Material and Methods

Chemicals: water at MS grade (18.5 MΩ) was purified with a MilliQ systems (Millipore, Milan, Italy). Solvents at HPLC grade, ammonium formate LC-MS grade, formic acid, TFA, human serum, acrolein, borohydride polymer supported, homocarnosine, and tyrosyl-histidine were purchased from Sigma Aldrich (Merck, Milan, Italy). L-carnosine, D-carnosine, carnosinol, balenine and anserine were gently provided by Flamma s.p.a. (Chignolo D'Isola, Bergamo, Italy).

PBS 1X without calcium and magnesium was purchased from Euroclone (Milan, Italy).

L-carnosine-d_3; D-carnosine-$^{13}C_3$ were synthetized in the laboratory of Prof. Dallanoce of the Department of Pharmaceutical Sciences, of the University of Milan.

Animal Tissue: animal tissue, serum and urine were collected form in vivo studies with the approval of the IACUC of East Carolina University, and by the Ethical Committee for Use of Animals of the Botucatu Medical School, São Paulo State, Brazil in compliance with the NIH's Guide for Care and Use of Laboratory Animals.

HPLCS-MS systems:

Experiments were carried out on two instruments:

a. LC-Exion100, coupled with API4000 mass spectrometer equipped with an ESI Turbo V source. Instrumental control carried out with the software Analyst v 1.6 (AB Sciex, Milan, Italy)

b. Dionex Ultimate 3000 nano LC coupled with an LTQ XL Orbitrap mass spectrometer equipped with either a Finningan ion max ESI source or a Finningan NSI-1 dynamic probe equipped with a stainless-steel emitter (5 cm length, OD 150 μM, ID 20 μM). Instrument were controlled with LTQ tune, Xcalibur v 4.0 and Chromeleon Xpress software (Thermo Fisher, Milan, Italy)

Preparation of Histidine dipeptides-RCS adduct:

HNE was obtained by acidic hydrolysis form the relative dimethylacetale synthetized as reported by Rees and colleagues [24]. Histidine dipeptides were incubated 1:1 with either HNE or Acrolein in 10 equivalent of phosphate buffer pH 7.4 at 37 °C for 3 hours. No signal related to the unmodified peptide was found in the solution by infusion analysis on LTQ XL Orbitrap instrument. An aliquot of the reaction mixture was further treated adding 10 equivalents of borohydride supported on polymer and the mixture reacted overnight at 37 °C. The reduced form of HNE and acrolein adduct with histidine dipeptides were obtained.

Tissue Homogenization:

Animal tissue homogenate were prepared at the final concentration of 0.1 g of tissue/ 1 mL PBS, by using a Bead Bug tissue homogenizer (Benchmark Scientific, USA). The homogenization was done at 4°C to avoid enzyme activation and degradation of the interested analyte.

3.2.2 Results and Discussion

HILIC chromatography for the direct separation of histidine dipeptides and RCS adducts.

The use of an Hypersil GOLD HILIC (2.1 x 150 mm, particle size 3 μm, pore size 175 Å) (Thermo Fisher, Milan, Italy) was evaluated. The column has a stationary phase based on polyethyleneimine chain. We evaluated several gradient conditions

starting from 95 % of organic solvent (i.e. acetonitrile) and buffers (ammonium formate).

To develop a quantitative method in LC-MS a triple quadrupole system (see section 3.2.1) was used, and the fragmentation ions of histidine dipeptides and RCS adducts were acquired. This was done in a semiautomatic mode. Collision energy parameter was optimized by the software for each transition (table 3.1).

Table 3.1*: Multiple Reaction Monitoring (MRM) transition of histidine dipeptides and RCS adduct; in bold the quantification transition.*

Compound	Precursor Ion (m/z)	Fragment ion (m/z)	Collision Energy (V)
carnosine	227.20	156.20	22.00
	227.20	**110.20**	35.00
anserine	241.30	170.30	25.00
	241.30	**109.30**	35.00
tyrosyl-histidine	**319.30**	**156.10**	25.00
	319.30	110.30	45.00
carnosinol	**213.20**	**166.20**	24.00
	213.20	107.10	30.00
homocarnosine	241.30	156.20	30.00
	241.30	**110.20**	35.00
carnosinol-HNE	**369.40**	**334.20**	34.00
	369.40	263.30	37.00
carnosine-HNE	**383.20**	**312.30**	32.00
	383.20	266.60	34.00

The gradient reported in table 3.2 was chosen in order have a good combination between retention time and analysis time. These are characteristics needed for quantitative analysis in many samples to improve the data output and reduce analysis time. The flow of the mobile phase was set at 250 µL/min and the column was thermostated at 40 °C. The mass spectrometer was working with the following source condition: +3.5 kV ionization potential, 25 units of curtain gas, 40 units of gas 1, 60 units of gas 2 heated at 550°C, and the detector in MRM mode.

During the analysis collision gas was set at 4 units, declustering potential at 45 units, entrance potential at 10 units, collision cell exit potential at 10 units and scanning time at 100ms for all the scans.

Table 3.2*: gradient method for the separation of histidine dipeptides and RCS derivatives on HILIC column.*

Time (min)	**A) ammonium formate 100mM pH 3(%)**	**B) acetonitrile (%)**
0.00	5.00	95.00
0.20	5.00	95.00
6.70	70.00	30.00
9.70	70.00	30.00
9.71	5.00	95.00
15.00	5.00	95.00

The method allows to have good retention time for all the analytes ($7<t_R<8.5$) and a good resolution of the peaks (figure 3.1). Since the fast gradient, some peaks can coelute, but the precursor ions or the MRM transition chosen allows the identification of the analyte (figure 3.2). Not more than 4 compounds per run where analyzed to have a low total scanning time and do not negatively impact the sensitivity. For each molecule at least two transitions were identified to characterize and quantify the analyte. (table 3.1).

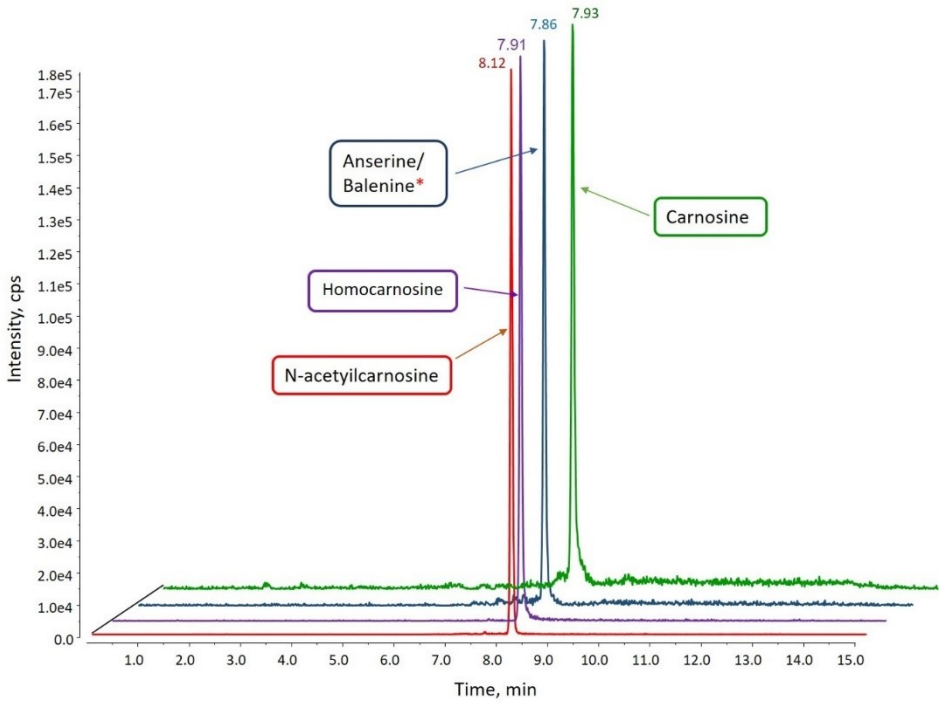

***Figure 3.1**: TIC of carnosine, anserine and balenine, n-acetylcarnosine, and homocarnosine*

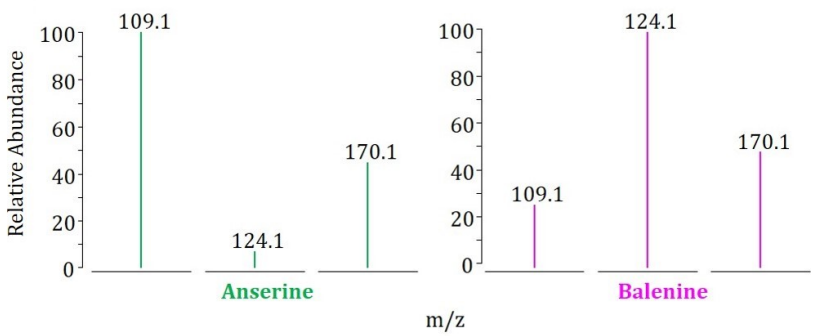

***Figure 3.2**: Different MRM transition intensity of anserine and balenine.*

Biospecimens preparation, and calibration curve:

The best sample preparation for the biological matrices was evaluated to have as less steps as possible and reduce sources of error.

The use of a HILIC column allows to inject a sample with a high percentage of organic solvents (i.e. ACN), solvent that can be also used to perform protein precipitation (i.e. PPR), hence removing proteins before the analysis.

A specific sample preparation was developed for each matrix type (figure 3.3):

- Serum is the easiest matrix to prepare since only a single step of deproteinization with 9 volume of ACN at 4 °C for 10 minutes is needed. After that, the sample is centrifugated at 14000 rpm for 10 minutes at 4 °C and the supernatant analyzed.
- Urine undergo the same sample preparation as serum with a centrifugation passage before the protein precipitation to separate the soluble components of urine from the insoluble one.
- Tissue samples were firstly homogenized as reported in section 3.2.1 The homogenate is then centrifugated at 14000 rpm for 10 minutes at 4 °C and the supernatant deproteinized before the analysis as reported for serum and urine matrices.

It is important to do all the sample preparation at 4 °C to reduce the activity of enzymes and prevent the degradation of analytes, especially if their enzymatic stability is not known.

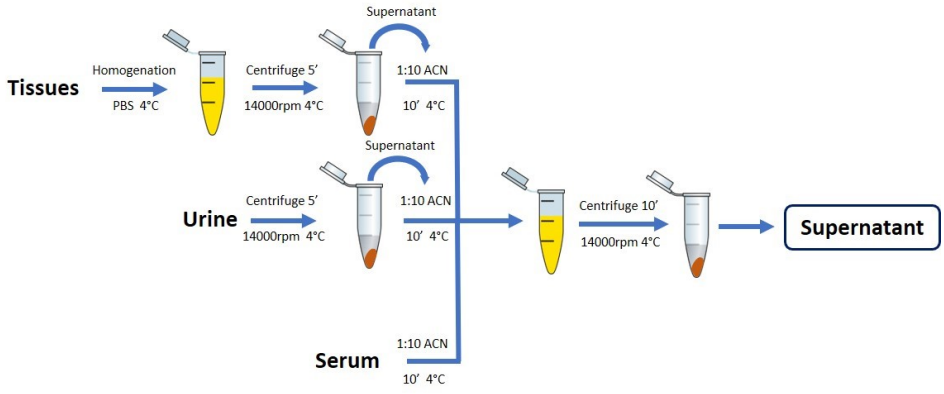

***Figure 3.3**: sample preparation scheme for the analysis of histidine containing dipeptides and derivatives in biospecimens (i.e. tissues, urine, and serum)*

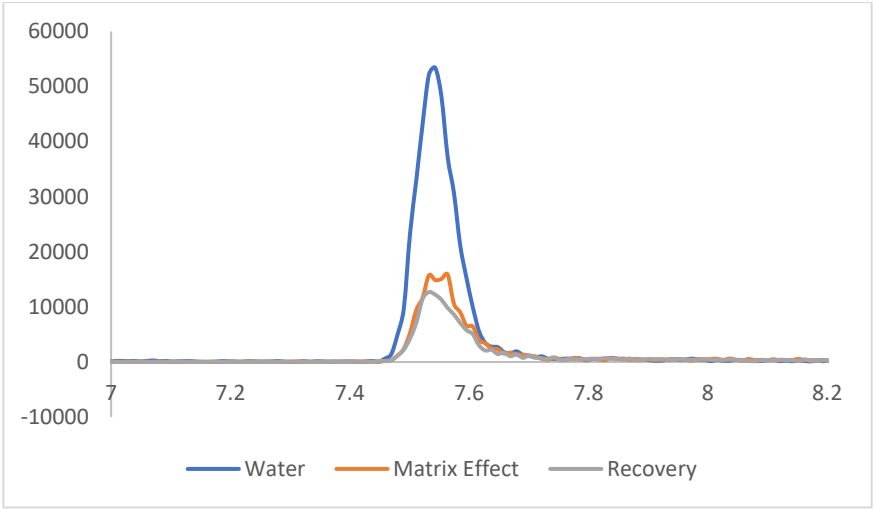

***Figure 3.4**: Carnosinol serum matrix effect and recovery of HILIC-ESI-MRM analysis (transition 216.2-166.2). Blue line: carnosinol in water; orange line: carnosinol added into PPR supernatants; gray line: carnosinol added to sample before PPR*

Figure 3.4 reports the chromatogram for carnosinol added to the serum matrix in different steps of sample preparation to evaluate the matrix effect and the recovery. By comparing the area under the curve of the peak of carnosinol in water and of carnosinol added to the deproteinized serum it is possible to calculate the matrix effect applying the following equation.

$$Matrix\ effect = 1 - \frac{AUC_{matrix}}{AUC_{water}} * 100$$

***Equation 3.1**: matrix effect. It indicates the interference effect of the matrix on the signal response of the analyte.*

In this example the matrix effect is of 70 %.

To calculate the recovery, equation 3.2 is applied. This is important to evaluate since the precipitation of the proteins can lead to the co-precipitation of the analyte and so reduce the amount of the molecule in the sample. It also indicates at which step the analyte should be added to set up the calibration curve.

$$Recovery = 1 - \frac{AUC_{before\ PPR}}{AUC_{After\ PPR}} * 100$$

Equation 3.2*: recovery. It indicates how much analyte is lost during sample preparation process (i.e. PPR).*

The recovery for the example reported in figure 3.4 is equal to 80%

In this case the analyte (i.e. carnosinol) is mostly affected by the matrix effect rather than the sample preparation process.

Based on this data, calibration curve in the interested matrices were set accordingly to the concentration range desired. The standard analyte was added directly to the matrix (e.g. urine, serum, tissue homogenate) and proteins were immediately precipitated to block enzymatic reactions. If the matrix was not free from the analyte a standard addition curve was applied. This method allows to have the calibration curve and to calculate the basal level of analyte present in the blank matrix. With this information the final concentration can be calculated accordingly.

In figure 3.5 is reported, as an example, the calibration curve of carnosinol in kidney tissue. The calibration curve is linear in a good range of concentration with a good R^2 value

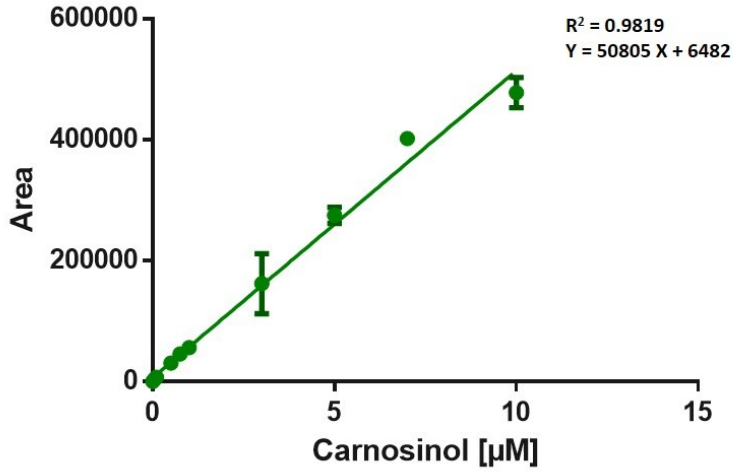

***Figure 3.5**: calibration curve of carnosinol in kidney*

Internal standard

To reduce the effect of the systematic and random error several internal standards were evaluated to find the most suitable for the analysis.

In literature the internal standard most used for carnosine quantification is the dipeptide tyrosyl-histidine [25, 26].

Nevertheless, for mass spectrometry analysis as reported in section 3.2.1 the ideal internal standard should be the stable isotope of the interested analyte. The main problem of these compounds is that they are not often available, and they are expensive. On the market is not possible to acquire any stable isotope of carnosine and the precursors for the synthesis are quite expensive.

Based on the market and funding availability we bought tri-deuterated histidine and β-alanine$^{13}C_3$. Thanks to the group of Prof. Dallanoce from the Department of Pharmaceutical Sciences of the University of Milan, the respectively carnosine molecule were synthetized (figure 3.6). In chronologic order we first used the tri-deuterated isotope and only recently we adopted the $^{13}C_3$ derivative.

Moreover, the $^{13}C_3$ derivative was synthetized as D-enantiomer to increase the enzymatic stability [23], allowing the addition prior protein precipitation process.

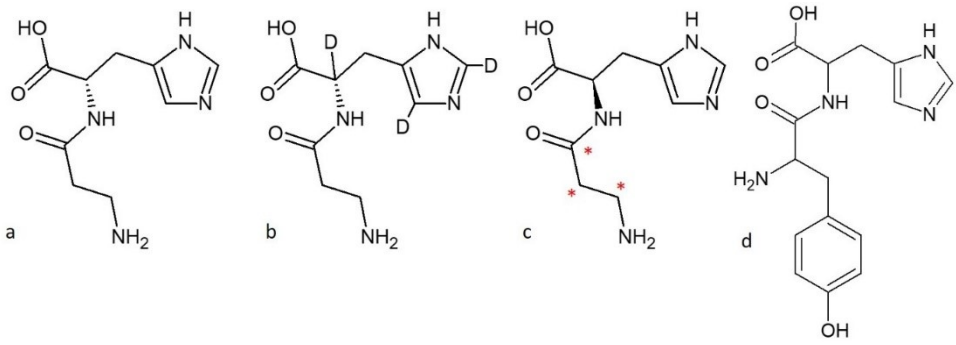

Figure 3.6*: structures of a) L-carnosine; b) L-carnosine-d3; c) D-carnosine13C3 d) tyrosyl-histidine*

The internal standard was added during the deproteinization process at a fixed concentration based on the calibration curve range. The differences between tyrosyl-histidine as internal standard or a stable isotope of carnosine (i.e. L-carnosine-d_3) were evaluated setting a calibration curve where each point was prepared in a different subject sample. In this way the matrix effect was amplify and it was possible to evaluate properly the effect of the internal standard.

In figure 3.7 is possible to see the calibration curve obtained with a) carnosine alone, b) carnosine corrected by tyrosyl-histidine, and c) carnosine corrected by L-carnosine-d_3.

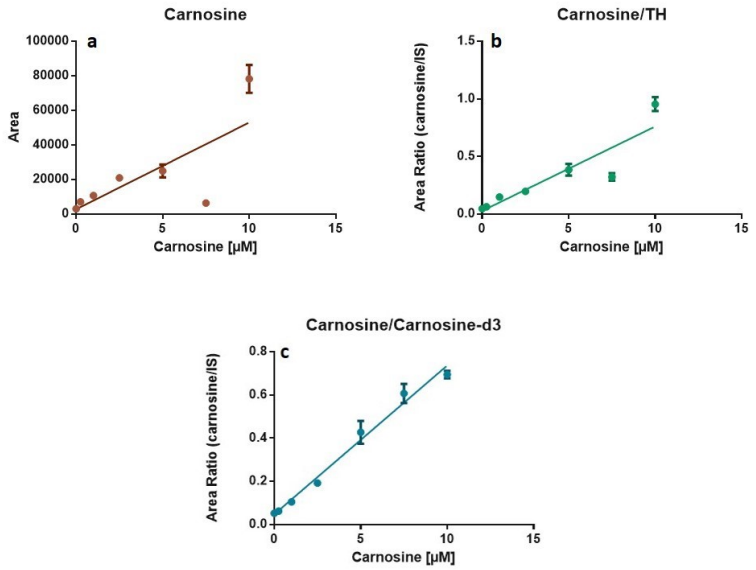

Figure 3.7*: calibration curve of carnosine in urine a) not corrected; b) corrected by TH; c) corrected by L-carnosine-d3*

Each subject has a matrix composition that is unique and interfere with the analyte in a unique way. If the AUC of carnosine is not corrected by any internal standard, it is not possible to have a proper linearity.

When tyrosyl-histidine is used to correct carnosine area, there is a slight increase in the linearity of the curve (a greater R^2), but the correction is not efficient, yet.

Only the use the stable isotope of L-carnosine-d_3 gave a real compensation of the matrix effect and a good calibration curve was obtained.

In this case the matrix effect was emphasized using matrix from different subject for each calibration curve point. A way to reduce the variability in the matrix effect for the calibration curve is the possibility to set it in a pool of blank matrices, where an average matrix effect will be present.

Recently the use of D-carnosine$^{13}C_3$ was implemented. This allow to add the internal standard also before the protein precipitation process since the D-enantiomer of carnosine is stable to the hydrolysis mediated by CN1. Moreover, since the isotopically stable building block to synthetized carnosine is the β-alanine, the synthetic route developed by the group of Prof. Dallanoce allows to synthetize all the analogue of carnosine and have a specific internal standard for each histidine dipeptide or derivative to be analyzed.

RP-nanoLC-HRMS method for the identification of histidine dipeptide-RCS adducts in biospecimens

Even if the LC-MS/MS method developed on the triple quadrupole is detecting the histidine-RCS adduct, we faced with the need of a more sensitive method able to determine them at very low amount in biological matrices. To do so a nano-LC chromatographic method was developed. The LC system was coupled to an LTQ-XL-Orbitrap detector as described in section 3.2.1. Using a nano system, we were able to increase the concentration of the analyte to the detector and the sensitivity using a high-resolution mass spectrometer.

The HILIC column used for the LC-MS/MS method is not available for nanoscale-LC system so we decided to use a reverse phase column. To have a good retention time for the RCS-adduct of histidine dipeptides a simil-2D system was developed. A first μ-Precolumn Cartridge (PrepMap100 C18, 0.3 x 5mm, 5 μm, 100 Å Dionex) was used as a guard to trap and concentrate the analyte and remove all the salts and too hydrophilic compound. For this purpose, an isocratic flow (10 μL/min) was used with 99% of water containing the 0.1% of TFA and 5 % of ACN with 0.1% formic acid. After 2 minutes the precolumn was diverted online to an Hypersil Gold Capillary Column (C18, 100 x 0.18 mm, 5 μm, 175 Å Thermo Scientific) at the flow rate of 1.5 μL/min following the gradient reported in table 3.3

***Table 3.3**: gradient method for the chromatographic separation*

Time (min)	A Water 0.1% FA (%)	B ACN 0.1%FA (%)
0.00	95	5
3.00	95	5
21.00	5	95
25.00	5	95
25.01	95	5
30.00	95	5

Ionization was performed in positive ion mode with 1.8 kV source voltage, 220 °C as capillary temperature, 35 V capillary voltage, 110 V tube lens. During the analysis the MS spectra were acquired in profile mode by Orbitrap using the following settings: scan range 250–700 m/z, 5×10^5 ions per scan, maximum inject time was set to 500 ms, resolution was set to 100000 (FWHM at m/z 400). Lock mass option was enabled to provide a real time internal mass calibration during the analysis using as reference a list of 20 abundant and known background signals already reported by Keller *et al*.[27] as common air contaminants in mass spectrometry.

Since a reverse phase chromatography has been used for this analysis, we add a further step to the sample preparation reported before. After the deproteinization, the supernatant was dried under vacuum with an RVC 2–18 rotational vacuum concentrator (Christ, Germany) and re-dissolved in TFA 1% aqueous solution, to have 2-fold final dilution of the tissue homogenates. TFA was also added in the sample to enhance the formation of the ion paring with the interested analyte. A large excess should be use when working with complex matrices, to saturate all possible positive counter ion and be sure that the analyte of interest interacts with it too.

As reported in section 3.1 the use of TFA in mass spectrometry is not good, but an escamotage to reduce the negative effect of the molecule on the mass spectrometer is using a simil-2D system.

The TFA is used as mobile phase only in the loading pump connected to the trap column. When the precolumn cartridge is divert online to the mass spectrometer, the mobile phase does not contain TFA and the only residual amount is the one in the sample. This amount is enough to have its effect on the retention time of hydrophilic analyte, but not to completely interfere with the ionization.

In figure 3.8 is possible to see the extracted ion current corresponding to the molecular ion 269.16082 of the standard carnosinol acrolein adduct (T_R = 10.33 min) in liver homogenate.

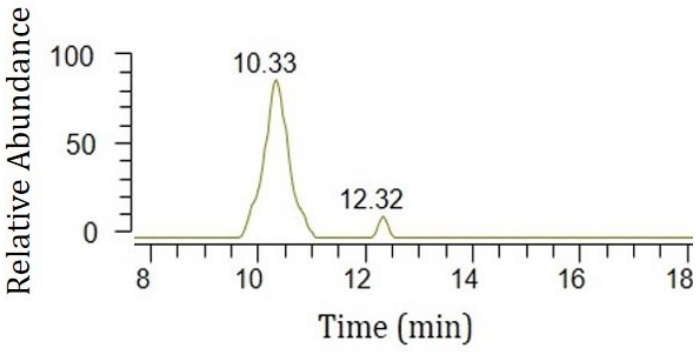

Figure 3.8*: extracted ion chromatogram of carnosine-acrolein adduct spiked in liver homogenates. Analyzed via RP-nanoLC-HRMS [M+H]+ = 269.16082 m/z ± 1 ppm; TR = 10.33 minutes*

3.2.3 Conclusions

We have developed a direct LC-MS/MS method for the separation and quantification of histidine dipeptides and the relative adduct with RCS. A proper sample preparation was developed to reduce the number of passages and the possible errors. Calibration curve were set in several matrices with a good linearity. The correct IS was evaluated and identified as the stable isotope of the interested analyte. Throughout the project two stable isotope were identified. L-carnosine-d_3 and the D-carnosine$^{13}C_3$.

The latter has more application since it can be added also in matrix itself since the D-enantiomer does not undergo to the hydrolysis mediated by CN1. Moreover, the building block for the synthesis is the beta-alanyl$^{13}C_3$, so any stable isotope derivatives of carnosine can be synthetized in an easier way.

To increase the sensitivity of the analysis and detect also very low amounts of analytes in complex matrices a nanoLC-HR-MS method was developed for the identification of histidine-dipeptides adduct with RCS. Even if the use of TFA was necessary since the separation occur on a reverse phase column, the simil-2D system allow to reduce the amount of TFA that reaches the source and give ion suppression problem. For this new method, the sample preparation protocol developed for the LC-MS/MS method is used with a small integration.

3.3 Development of a direct method for the determination of serum carnosinase activity.

3.3.1 Material and Methods

Chemicals: as reported in section 3.2.1

Sample Preparation: serum was diluted down to the desired concentration with 10 mM phosphate buffer saline (PBS, pH 7.4). The incubation started by spiking the analytes into the serum sample down to 50 μM final concentration. Control samples were prepared by blocking the hydrolysis at the incubation starting point, hydrolyzed samples by blocking the hydrolysis at the reaction endpoint. Small adjustments concerning serum dilution and endpoint sampling time were necessary for each substrate to result in a residual amount suitable for the determination of the hydrolysis rate. Preliminary data indicated that a serum dilution ranging from 1:1 to 1:10 was not impacting on the measurement of carnosine hydrolysis rate and the most reproducible data were obtained when the incubation endpoint resulted in a substrate between 50–75 % of its initial concentration.

We therefore optimized the protocol to keep an incubation endpoint of 10 min when carnosine was used as substrate, whereas 30 min were necessary for anserine, balenine and homocarnosine. Serum dilution was 1:10 to test the hydrolytic activity towards carnosine and anserine, whereas a 1:1 dilution was used for balenine and homocarnosine. The hydrolysis was blocked at the desired time points inducing protein precipitation at 4 °C by the addition of 9 volumes of ACN containing a fixed amount of internal standard (L-carnosine-d_3). Before the analysis samples were centrifugated at 4 °C for 10 minutes at 14000 rpm and supernatant was collected in glass autosampler vials. Calibration standards were prepared from aliquots of serum, each one spiked with a different amount of analyte, whereas the amount of internal standard and sample preparation procedure remained the same as described above.

LC-ESI-MS/MS method: as reported in section 3.2.1

3.3.2 Results and Discussion

Figure 3.9 reports the chromatograms of 50 µM carnosine spiked into 10-fold diluted serum. Samples are referred to control sample (figure 3.9-a) and to hydrolyzed sample (figure 3.9-b), which were deproteinized at the beginning and at the endpoint (i.e. 10 minutes) of the incubation, respectively.

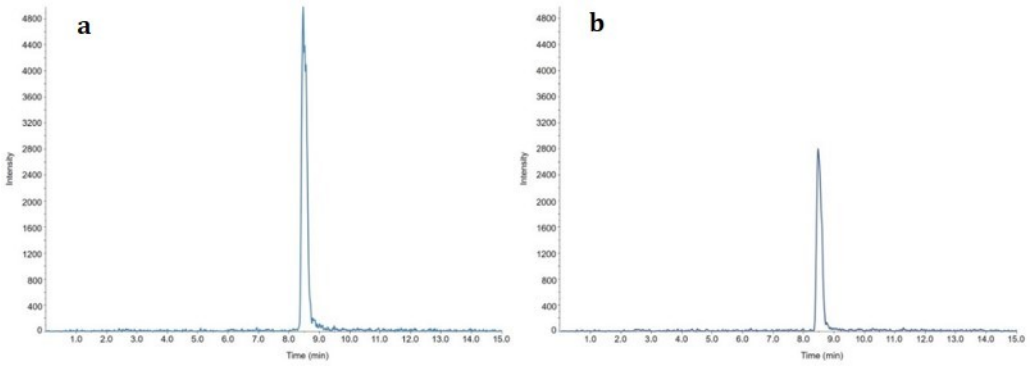

Figure 3.9*: Extracted ion chromatogram of transition 227.2-110.2 m/z for carnosine in serum sample at a) the beginning and b) the endpoint of the incubation. Analyzed with the HILIC-ESI-MRM method.*

To calculate the serum hydrolytic rate of carnosine in our samples we applied the equation 3.3

$$HR = \frac{(nmol_{CTR} - nmol_{HYD})}{V \times t}$$

Equation 3.3*: hydrolytic rate. It indicates the hydrolysis rate as nanomole of substrate consumed in one hour by one microliter of serum*

Where HR is serum hydrolytic rate measured as nanomoles of carnosine hydrolyzed in one hour by one µL of serum (nmol/(µL*h)); $nmol_{CTR}$ are the nanomoles of carnosine as determined in the control sample; $nmol_{HYD}$ are the residual nanomoles of carnosine as determined in the hydrolyzed sample and V is the volume of serum (in µL) and t is the incubation time (in hours).

The nanomoles of substrate in the control and hydrolyzed samples were determined by isotope dilution mass spectrometry (IDMS). In detail, a calibration curves reporting the area ratio between substrate and the internal standard as function of the amount of substrate injected were obtained from the analysis of calibration standard triplicates. The method was qualified for substrate quantification by evaluating the specificity, linearity, precision, and accuracy (table 3.4).

***Table 3.4**: calibration curve of carnosine, anserine, homocarnosine and balenine in serum, analyzed with the HILIC-ESI-MRM method.*

ANALYTE CONCENTRATION (μM)	1	5	10	20	40	60
CARNOSINE						
PRECISION (CV%)	6.74	3.14	3.28	3.32	1.53	0.87
ACCURACY (ER%)	5.04	-8.73	0.65	0.37	1.77	2.14
RECOVERY (%)			98.80		107.09	
MATRIX EFFECT (%)			82.03		68.19	
LIMIT OF DETECTION (LOD)	X					
LOWEST LIMIT OF QUANTIFICATION (LLOQ)	X					
LINEARITY RANGE	1-60 μM; $y = 0.0367x + 0.0474$; $R^2 = 0.9964$					
ANSERINE						
PRECISION (CV%)	5.54	3.81	1.81	2.56	3.73	4.05
ACCURACY (ER%)	-0.19	1.51	-0.50	-0.03	-1.67	0.88
RECOVERY (%)			98.78		95.84	
MATRIX EFFECT (%)			90.64		89.74	
LIMIT OF DETECTION (LOD)	X					
LOWEST LIMIT OF QUANTIFICATION (LLOQ)	X					
LINEARITY RANGE	1-60 μM; $y = 0.0492x - 0.0049$; $R^2 = 0.9983$					
HOMOCARNOSINE						
PRECISION (CV%)	1.09	1.91	1.63	2.74	4.44	2.29
ACCURACY (ER%)	1.12	-4.64	-3.78	0.94	-1.32	7.67
RECOVERY (%)			105.62		100.58	
MATRIX EFFECT (%)			71.70		76.97	
LIMIT OF DETECTION (LOD)	X					
LOWEST LIMIT OF QUANTIFICATION (LLOQ)	X					
LINEARITY RANGE	1-60 μM; $y = 0.1775x - 0.0039$; $R^2 = 0.9969$					
BALENINE						
PRECISION (CV%)	24.15	1.79	12.57	17.61	3.90	8.03
ACCURACY (ER%)	-0.73	6.70	-3.99	-7.64	8.02	-2.36
RECOVERY (%)			109.09		108.50	
MATRIX EFFECT (%)			79.90		89.26	
LIMIT OF DETECTION (LOD)	X					
LOWEST LIMIT OF QUANTIFICATION (LLOQ)		X				
LINEARITY RANGE	1-60 μM; $y = 0.1585x - 0.0014$; $R^2 = 0.9767$					

The hydrolytic rates of homocarnosine, anserine and balenine were determined with the same protocol described for carnosine but monitoring different MS transitions.

The hydrolytic rates as measured by our assay are reported in table 3.5.

Table 3.5*: serum hydrolytic rate of natural histidine dipeptides*

Peptide	Concentration (μM)	Serum hydrolytic rate (nmol/(h*μL))
Carnosine	50	1.328 ± 0.081 (N=3)
Homocarnosine	50	0.017 ± 0.001 (N=3)
Anserine	50	0.495 ± 0.036 (N=3)
Balenine	50	0.050 ± 0.001 (N=3)

To verify method reliability, we compared our experimental results with data reported in literature. Our method seems reliable since the activity falls in the range already reported by Peters et al., who spiking 80 μM carnosine into serum determined a serum hydrolytic rate ranging from 0.9-1.3 nmol/(h*μL) [28].

In the same paper hydrolytic rates ranging from 0.9 to 3.1 nmol/(h*μL) have been reported for starting concentrations of carnosine between 80 μM and 480 μM. Such experiments were mostly done with serum for single donors, whereas our serum was a commercially available pool of serum from AB donors. Since the hydrolytic activity depends serum initial content [29] but is also influenced by the substrate initial concentration [28], a further validation of our method was done by measuring carnosine hydrolysis rate at increasing substrate concentration up to enzyme saturation (figure 3.10).

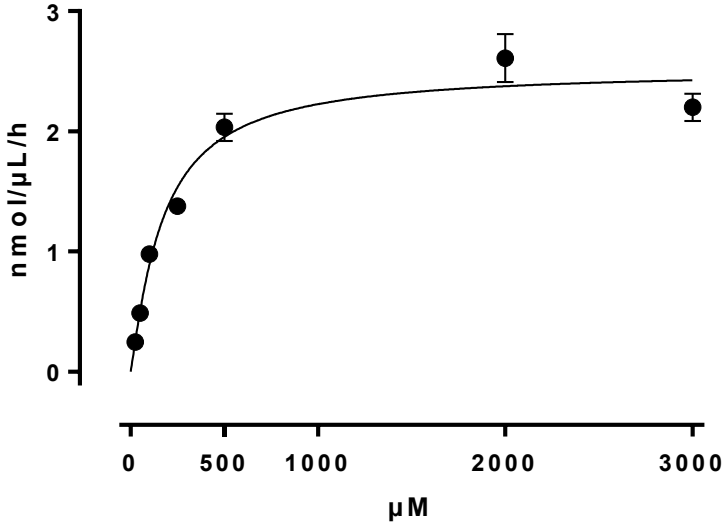

Figure 3.10*: Plot of serum hydrolysis rate at different concentration of carnosine*

The best fitting curve (R^2 of 0.9634) obtained using a Hill equation gave a Hill coefficient (h) of 1.152±0.18 (i.e. an almost Michaelis-Menten curve), whereas 2.513±0.14 nmol/(h*µL) and 169.3±29.04 µM were the estimated values for Vmax and Km, respectively. Such findings are consistent with previously reported data even if our measurements were performed in whole serum and not on purified recombinant enzyme [28, 30]. This implies that our method gives a reliable measurement of carnosine hydrolysis rate over a broad range of carnosine concentrations.

Moreover, since the saturation curves as obtained in whole serum and purified enzyme are superimposable, CN1 is the sole or at least the main contributor to the overall hydrolysis rate as observed in the pooled serum sample we used.

Concerning the measurement of the hydrolysis rate of natural carnosine analogs (i.e. anserine, balenine and homocarnosine), an article of 2011 reported the values as measured by fluorimetry for some of the peptides we tested [31]. Such values are reported in table 3.6.

Table 3.6*: serum hydrolytic rate of natural histidine dipeptides as reported by Peters et al [31].*

Peptide	Concentration (µM)	Serum hydrolytic rate (nmol/(h*µL))
Carnosine	1000	3.2 ± 0.9 (N=94)
Homocarnosine	1000	0.065 ± 0.04 (N=105)
Anserine	1000	0.015 ± 0.02 (N=106)

For any given peptide, the hydrolysis rate as reported in literature (table 3.6) or measured by our assay (table 3.5) looks quite different. As already mentioned, such differences can be due to substrate concentration or enzyme serum concentration [29, 31]. Looking at the results reported in literature or obtained by our assay, they have been done by using different matrices (serum from single subject vs pooled serum), in different condition (1000 µM vs 50 µM substrate) and resulted to have different precisions and absolute values of hydrolysis rate. Therefore, to overcome the problem of comparing data obtained in inconsistent experimental conditions, we compared peptide relative velocities (i.e. ratio of the hydrolysis rates between a given peptide and carnosine). As reported by Hayya et al., the relative velocities can be calculated as mean± standard deviation from the values reported in tables 3.5 and 3.6 using second-order Taylor series expansions [32].

According to data reported in literature, homocarnosine relative velocity is 2.19 % ±1.37, which is consistent with our findings (i.e. 1.31 % ±0.09). On the contrary, we observed a discrepancy on anserine relative velocity, which was 0.49% ±0.12 according to literature data, while a value of 37.45 %± 3.53 can be calculated from our data. However, our findings look more consistent with recent experiments of Everaert et al. [33], who reported that compared to anserine carnosine has a threefold shorter half-life in human serum (6 vs 18 minutes at 100 µM starting concentration).

Since literature data have been collected by fluorimetry upon OPA derivatization and such a reaction involves the imidazole ring, we can speculate that derivatization can be ineffective for anserine leading to an underestimation of the reaction product. Conversely, the LC-MS assay we developed cannot suffer of such a bias since it provides a direct measurement of the substrate residual concentration. Finally, we were able to determine the hydrolysis rate in serum also for balenine, which have yet not been reported. Specifically, we determined a relative velocity of 3.81% ±0.23 suggesting that such a peptide is hydrolyzed slightly faster than homocarnosine, but tenfold slowly than its regioisomer anserine.

3.3.3 Conclusions

Herein, we reported a new and direct method for the measurement of carnosinase activity relying on liquid chromatography-tandem mass spectrometry. The method provided a reliable and validated measurement of hydrolysis rate for carnosine and its natural analogs. The rate of hydrolysis was obtained by a direct quantitation of the time-dependent substrate consumption in serum using the isotope dilution method (i.e. L-carnosine-d_3 as internal standard). For carnosine hydrolysis kinetics, the method was suitable also for the determination of Vmax and Km through enzyme saturation experiments. Consistently with literature data, our findings suggest that homocarnosine is hydrolyzed at 2% of carnosine hydrolysis rate. Conversely, we found a faster hydrolysis rate for anserine compared to some literature data, although our experimental data looks more in agreement with the serum half-life of anserine as reported by other authors and are supported also by computational studies [34]. Moreover, the method allowed to determine for the first time the hydrolytic rate of balenine in serum, which resulted to be about tenfold slower than its analog anserine. Since we worked on pooled serum, such data don't allow to determine whether anserine, balenine and homocarnosine hydrolysis in serum is solely due to CN1 activity.

However, the method herein reported is suitable for the specific measurement of CN1 activity using purified or recombinant enzyme

3.4 Development of a LC-UV-MS method for the simultaneous separation of carnosine enantiomers and natural derivatives.

3.4.1 Materials and Methods

Chemicals: see section 3.2.1

HPLC-UV-MS system: The platform consisted of a Surveyor HPLC system (Thermo Scientific, Milan, Italy) including a MS quaternary pump which delivered a flow rate of 1.0mL/min for all the experiments. Eluents made up of water containing 0.1% (by volume) formic acid (eluent A) and methanol (eluent B) were used. Different eluent compositions were tested, using binary mixtures of eluents A and B with a methanol percentage ranging from20% (v) to 50% (v). Eluents were degassed by the pump vacuum degasser before being mixed. Surveyor autosampler provided automatic injections of 10 µL sample aliquots. The chromatographic separation was provided by a Chirobiotic T column (250 x 4.6 mm, 5 µm particle size, Sigma-Aldrich, Milan, Italy). The column eluate was split with a T junction both to an UV-PDA Plus detector and a TSQ quantum ultra, triple quadrupole mass spectrometer (Thermo Scientific, Milan, Italy). A Finnigan IonMax electrospray ionization source (ESI) assembled witha32-gauge stainless steel emitter (Thermo Scientific, Milan, Italy) was used for spraying the eluate into the mass spectrometer by applying 3.5 kV spray voltage, 80 units of sheath gas, 20 unit of auxiliary gas, and 270 °C capillary temperature.

The triple quadrupole detector was working in positive ion mode to monitor the MRM transition reported in table 3.7.

***Table 3.7**: MRM transition used for monitoring anserine, balenine and carnosine enantiomers*

	Precursor Ion (m/z)	Parent Ion (m/z)	Collision Energy (V)
Carnosine Enantiomers	227.20	210.10	14
	227.20	156.20	20
	227.20	110.20	27
Anserine/ Balenine	241.30	170.30	17
	241.30	124.10	23
	241.30	109.30	26

Fragmentation was provided by collision with argon at the pressure of 1.5 Torr, all the other parameters were optimized in a semiautomatic mode, directly injecting a standard solution of the analytes.

Instrumental control and data analysis were provided by TSQ Tune and Xcalibur 4.0 (Thermo Scientific). Peak fitting was done using the gaussian fitting function of OriginPro 2017 (OriginLab Corporation, Northampton, Ma, USA).

3.4.2 Results and Discussion

To obtain the separation of L-carnosine and D-carnosine, with the help of the group of Prof. Sardella of the University of Perugia, a suitable chromatographic separation was developed using a teicoplanin-based column. The choice was done since the teicoplanin-based column is recognized to be highly effective in the separation of amino acid and peptides [35, 36]. Several mobile phases were tested to identify the suitable conditions. The final choice was done on 0.1% of formic acid (pH 3.1) and methanol. This combination of mobile phase was chosen since it is suitable for the hyphenation of the chromatography system to the mass spectrometer detector [37].

Based on these preliminary data we tested the opportunity of setting up a chromatographic method allowing not only the simultaneous separation of carnosine enantiomers but also L-carnosine analogs (i.e. L-anserine and L-balenine).

Figure 3.11 reports the LC-UV chromatograms obtained from the analysis of mixtures containing anserine, balenine and both carnosine enantiomers at equimolar concentrations

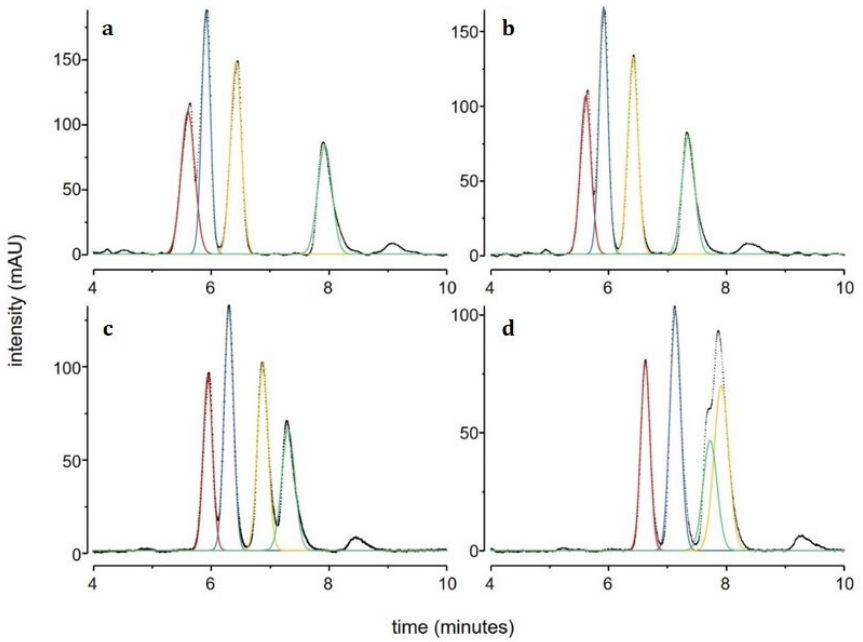

Figure 3.11*: Separation of carnosine analogs on the Chirobiotic T column with aqueous formic acid mixed with 50% (A) or 40% (B) or 30% (C) or 20% (D) methanol. Black dots for experimental points, colored lines for the best gaussian fitting for the peak of L-carnosine (red), anserine (blue), balenine (yellow) and D-carnosine (green).*

The column appears to be suitable for the separation of the peptide mixture even with isocratic conditions. As previously reported [37], D-carnosine is more retained than the L enantiomer in such a column, but the most interesting finding is that anserine and balenine generate distinct peaks with the two peaks of carnosine enantiomers.

For most of the methods tested, the elution order was L-carnosine followed by anserine, followed by balenine then D-carnosine, while the reduction of methanol in the mobile phase down to 20% (v) produced an inversion of retention order between balenine and D-carnosine.

Resolution (R_S) between each pair of adjacent peaks was calculated by applying the formula

$$Rs = \frac{2|t1-t2|}{(W1+W2)}$$

Equation 3.4: resolution. It indicates the degree of separation of two adjacent peaks

t1 and t2 being the elution time of the peaks, W1 and W2 being the peak base width calculated as 4σ from the gaussian fit.

Table 3.8*: Peak resolution (RS) as a function of mobile phase. * inversion of elution order.*

peak pair	Methanol in the mobile phase (%)			
	50	40	30	20
	R_S			
L-carnosine/D-carnosine	4.94	4.41	3.62	2.79
L-carnosine/anserine	0.81	0.92	1.13	1.50
L-carnosine/balenine	1.97	2.27	2.81	3.19
anserine/balenine	1.53	1.55	1.72	1.82
balenine/D-carnosine	3.56	2.43	1.09	0.40*
anserine/D-carnosine	5.20	3.96	2.65	1.41

Results reported in table 3.8 prove that the Chirobiotic T column allows resolution for carnosine enantiomers no matter what the percentage of methanol used for the elution. In fact, R_S between L-carnosine and D-carnosine is always exceeding 1.5, which is the value expected for two completely resolved peaks.

However, the most interesting result is that the method allows also regioselectivity, since with all the condition tested the peak pair of the positional isomers anserine and balenine resulted to have a R_S exceeding 1.5, with a trend of an increment of the peak resolution with lower methanol percentages. Additionally, the column allows also remarkable chemoselectivity and chemoresolution at low methanol percentages. Specifically, the calculated R_S between the peak pair L-carnosine/balenine is exceeding 1.5 for all tested conditions, while for the peak pair L-carnosine/anserine the R_S exceeds 1.5 only when 20% methanol is used. The use of 20% methanol leads to an inversion of retention between D-carnosine and balenine with partially overlapped peaks (D-carnosine/balenine R_S of 0.4) and loss of resolution from anserine (anserine/D-carnosine R_S of 1.4). This evidence suggests an active participation of eluent components in the analyte-stationary phase binding mechanism. Indeed, mobile phase should not be regarded as a passive transporter of the analytes across the column, but it is rather involved in defining the conformational features of the selector and selectands. On the basis of all the above, the use of 30% methanol better compromised with such effects and leads to an enantio-, chemo- and regioselectivity. In such condition all peak pairs are sufficiently resolved (R_S>1), although for L-carnosine/anserine and balenine/D-carnosine peak pairs the separation is not complete (R_S<1.5).

This problem can be easily resolved by using a more selective detector such as a mass spectrometer. In fact, another advantage of the developed method is the use of volatile eluents which extends its application also to studies with MS detectors operating with electrospray [37].

Figure 3.12 demonstrates that the incomplete resolution of the L-carnosine/anserine and balenine/D-carnosine peak pairs is not a problem when a mass spectrometer is used, since the molecular weight differences between peptides allows their selective determination.

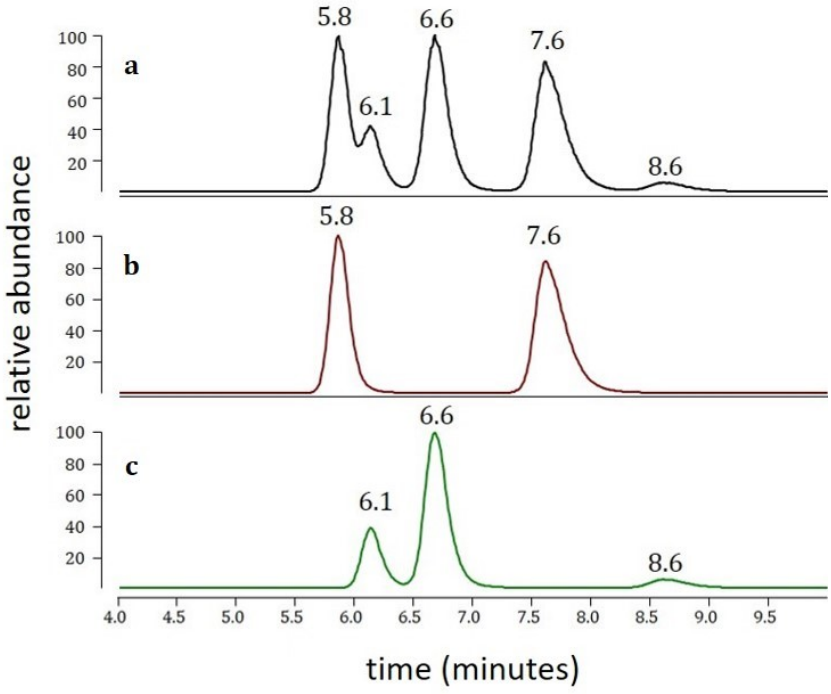

Figure 3.12*: Separation of an equimolar mixture of carnosine analogs on the Chirobiotic T column with aqueous formic acid mixed with 30% Methanol. A) Total ion chromatogram, B) MRM chromatograms for carnosine transitions or C) anserine and balenine transitions*

The MRM trace in figure 3.12-b is reporting two peaks since carnosine enantiomers have the same fragmentation pattern (figure 3.13). On the other hand, MRM trace 3.12-c is selectively reporting the anserine and balenine peak pair, since common fragments were selected for the analysis, although such peptides have slightly different patterns in term of the relative abundance and m/z values of some fragment ions (figure 3.13).

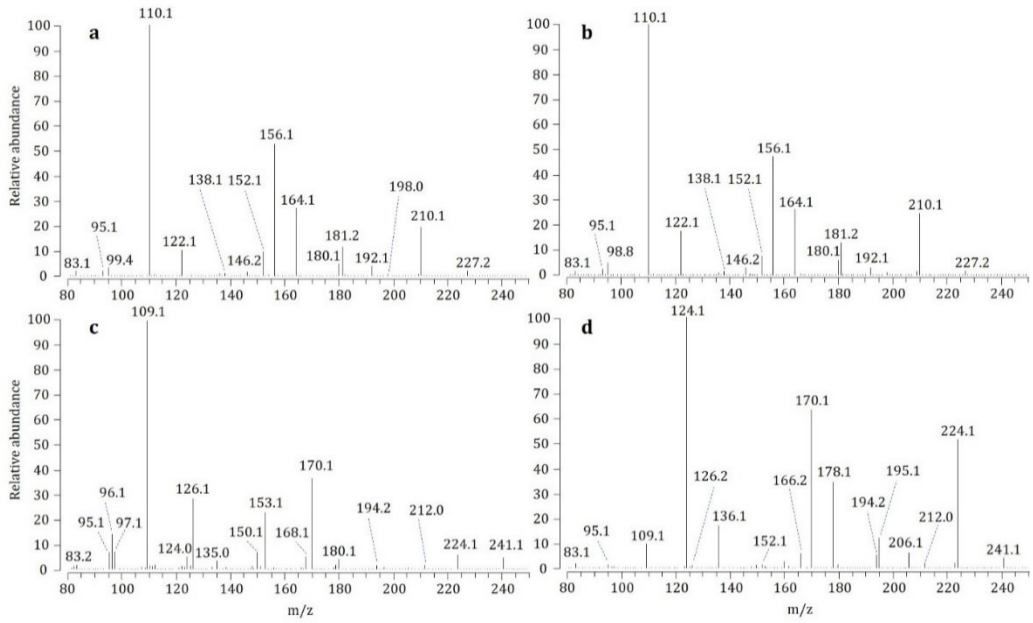

Figure 3.13*: Fragmentation spectra of a) L-carnosine, b) D-carnosine, c) anserine and d) balenine.*

This implies that fragment ions with different m/z values can be potentially considered to build selective MRM methods to monitor either anserine or balenine, even if chromatographic separation has insufficient peak resolution.

Notably, all chromatograms in figure 3.12 have an additional peak eluting at 8.6 minutes. This peak is undetectable in the MRM method built with carnosine transitions but persists in the MRM trace built with anserine and balenine transitions. Interestingly, as reported in figure 3.14, the fragmentation spectrum of such a peak is consistent with balenine spectrum.

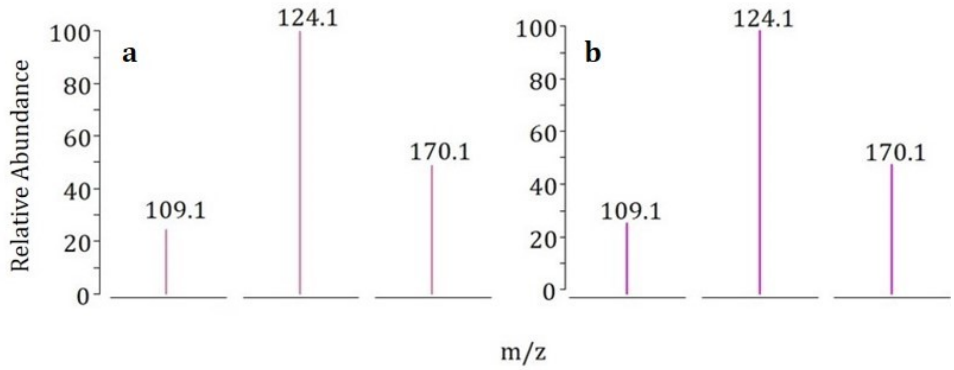

***Figure 3.14**: Intensities of MRM fragments for the impurity eluting at Rt 8.6 minutes (spectrum A) and balenine (Rt 6.6 minutes, spectrum B)*

Notably, relative retention time between the peaks of the impurity and balenine is similar to the relative retention time between carnosine enantiomers (figure 3.12-b). The impurity peak was therefore attributed to D-balenine, since the synthesis of balenine standard was not designed to ensure racemization, according to the supplier indication.

Overall the method herein reported permits the identification of carnosine analogs in food or food supplements, with an additional advantage of allowing the detection of impurities generated by peptide racemization, which is the real novelty compared to available methodologies [11, 38].

3.4.3 Conclusions

Herein, we demonstrated that a Chirobiotic T column can be used for developing enantio-, regio- and chemoselective method for monitoring carnosine analogs in food or food supplements.

The method resulted to be flexible for the use of both UV detectors and mass spectrometers. The advantages provided by mass spectrometry in term of sensitivity and selectivity were investigated using complex mixtures of carnosine analogs, even for methods with suboptimal chromatographic separation.

The results suggest that the developed chiral HPLC method is a powerful tool for monitoring the purity of carnosine-containing foods/food supplements.

3.5 CONCLUSIONS

During this project, a direct and easy LC-MS/MS method was developed for the identification and quantification of histidine dipeptides and RCS-adducts in biospecimens. The method was developed to suit the final purpose of directly detecting histidine dipeptides and derivatives in biospecimens, avoiding the use of TFA in the mobile phase. To do this a hydrophilic interaction chromatography (HILIC) was employed. Thanks to the hyphenation of mass spectrometry it is possible to increase the selectivity of the analysis especially by introducing the multiple reaction monitoring mode. Based on this new chromatographic method it was possible to develop a fast and easy sample preparation of biological matrices. Reducing the number of passages in the sample preparation helps in the reduction of systematic error in the analysis. The method was applied successfully to several matrices and the suitable internal standard was identified. Since the ionization process in mass spectrometry is susceptible to several factors, the stable isotope of the interested analyte was identified as the best internal standard. Thanks to the lab of Prof. Dallanoce we were able to first have L-carnosine-d_3 that was fully implemented in the LC-MS/MS method. Only recently the stable isotope D-carnosine $^{13}C_3$ was synthetized and integrated too. This new IS allows the use as early as possible in the sample preparation since D-carnosine is not susceptible to the hydrolysis mediated by CN1. Moreover, since the carbon isotopes are on the β-alanine moiety, it is possible to synthetize all the possible natural analogues of carnosine in an easier way.

A more sensitive method was developed for the identification of low amount of analyte. A simil-2D nanoLC-HR-MS method allows to exploit the potentiality of the TFA in increasing the retention time of hydrophilic compound in reverse phase chromatography, without facing with the full drawbacks of using such a molecule in mass spectrometry (i.e. ion suppression).

This, coupled with the increase sensitivity of both a nano HPLC and a high-resolution mass spectrometry, were successfully for the identification of low amount of histidine dipeptides-RCS adduct in biospecimens.

A combination of a tailored degradation kinetic in human serum and the LC-MS/MS method was developed to directly measure the serum hydrolytic rate of carnosine and the other natural analogues. The method was validated in comparison with data present in literature and it results to be reliable and accurate. We determined that CN1 is the main, if not the only, enzyme responsible for the metabolism of carnosine in human serum and for the first time we determined the serum hydrolytic rate for the carnosine analogue balenine.

Carnosine properties are well known in several scientific field and food supplement based on carnosine are on the markets. Most of the biological effects are done by the L-enantiomer of carnosine since the D-enantiomer is not bioavailable, not being absorbed in the gastro-intestine tract. Therefore, an enantioselective chromatography was developed for the separation of L and D carnosine. It was also implemented for the separation of anserine and balenine, the other two main natural histidine dipeptides. The chromatography is coupled with both UV and MS detector and it allows to fully resolve the 4 analytes with an isocratic method. Moreover, the use of mass spectrometry increases the sensitivity of the method and the selectivity, letting the recognition of putative enantiomeric impurity of all the analytes.

Analytical methods are powerful tools that can answers to several question researchers have. It is important that enough time is spent to understand the methodology in term of chromatographic separation, detector suitability and sample preparation. Mass spectrometry have changed the world giving new possibility to the analytical field. It is as powerful as difficult to handle; hence a good understanding of the technique and the application is necessary.

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4 Histidine dipeptides react with RCS in animal models

4.1. INTRODUCTION

The reactivity of carnosine with reactive carbonyl species is already known and well characterized *in vitro* [1, 2]. Although carnosine activity has been demonstrated in animal models of obesity [3], diabetes [4], ischemia/reperfusion [5, 6], cancer [7], neurodegeneration [8, 9] and atherosclerosis [10] only a few pilot clinical studies on humans focused on obesity [11, 12] and diabetes [13] have been carried out. Unfortunately, most of these studies were neither focused on characterizing the metabolism of the supplemented carnosine, nor on the identification of carnosine-RCS adducts, as proof of the ability of the peptide to deactivate RCS in vivo. Very few studies identify carnosine-RCS adduct in animal matrices such as serum and urine [3, 4] and even less in humans [12, 14].

Hereafter, a deep investigation of the mechanism of action of carnosinol in animal model of metabolic syndrome is carried out. Carnosinol was administrated in drinking water at a final dose of 45 mg/kg to wild type (wt) mice and GPx4 +/- mice fed with high fat high sugar (HFHS) diet. The expression and activity of the selenoenzyme GPx4 (Glutathione Peroxidase 4, EC 1.11.1.12) is known to be intimately involved in the regulation of disease involving lipid peroxidation process. As 1 of only 3 antioxidant enzymes essential for development [15], GPx4 has recently been the target of intense scrutiny by investigators. In particular, a critical role for GPx4 in regulating ferroptosis and subsequent organ failure has been reported [16–19]. Furthermore, genetic variants of GPx4 that result in diminished activity and/or enzyme content are associated with obesity and cardiovascular disease in humans [20–22]. It has reported that GPx4-deficient (GPx4+/–) male mice acquire severe insulin resistance, steatohepatitis, and cardiomyopathy on a high fat high sugar (HFHS) diet and that diabetic patients have diminished GPx4 content and elevated HNE adduct levels in their heart tissue compared with levels in age-matched nondiabetic patients [23]. In the present study, it has been used male GPx4+/– mice as a model to pharmacologically assess carnosinol in a context of enhanced lipid peroxidation–induced by obesity.

As shown in figure 4.1, GPx4+/– mice had greater levels of HNE adducts in skeletal muscle (Figure 4.1-a, b) and pancreas (figure 4.1-c, d) following a HFHS diet compared with levels in WT mice. Carnosinol was effective at mitigating HNE adducts in these organs from both WT and GPx4+/– mice. Moreover, beside dose-dependently attenuate HNE adduct formation in liver and skeletal muscle, carnosinol simultaneously mitigating inflammation, dyslipidemia, insulin resistance, and steatohepatitis. These improvements in metabolic parameters with carnosinol were not due to changes in energy expenditure, physical activity, adiposity, or body weight [24].

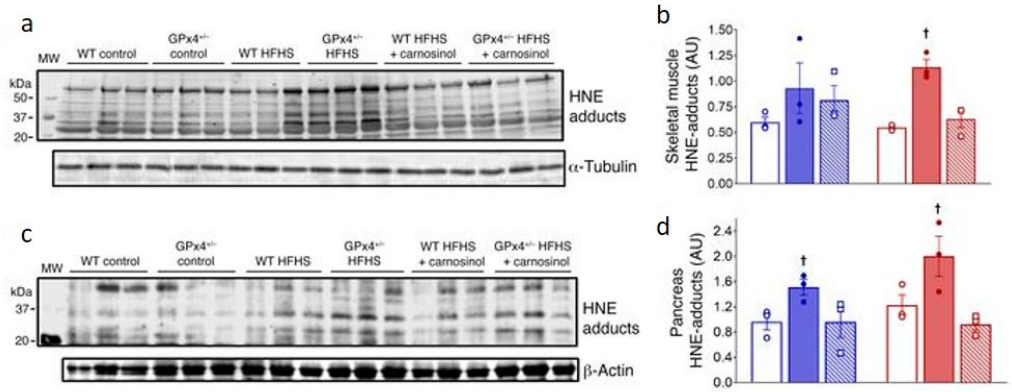

Figure 4.1*: Representative immunoblots for HNE adducts in whole-tissue homogenates prepared from mixed gastrocnemius skeletal muscle (a) and pancreas (c) tissue (n = 3 mice per group), along with the corresponding densitometric analysis (b and d). † P < 0.01 versus control diet within each respective genotype determined. A 2-way ANOVA followed by Tukey's multiple comparisons test was used to test for the main effect of the treatment within each genotype. Edited from Anderson et al [24]*

4.2. MATERIAL AND METHODS

Chemicals, sample preparation, synthesis of carnosinol-HNE and carnosinol-acrolein adduct are reported in chapter 3.2.1

For carnosinol and carnosinol-HNE quantification it was used the HILIC-ESI-MRM method reported in chapter 3.2.2, whereas for carnosinol-acrolein it was used the nanoLC-HRMS method reported in chapter 3.2.2.

Study approval. All in vivo studies were performed in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in the United States. Studies were performed with the approval of the IACUC of East Carolina University, in compliance with NIH guidelines for the care and use of laboratory animals [24].

4.3. RESULTS AND DISCUSSION

6.3.1. Carnosinol distribution in tissues

Carnosine reacts with HNE through an autocatalytic mechanism as reported in figure 4.2. First a Schiff base is formed by the interaction of the carbonyl group of HNE and the primary amine of carnosine. The Schiff base present a favorable conformation for the nucleophilic attack of the nitrogen (π) of the imidazole ring of carnosine to the partial positive charge in position β-position of the imine through a Michael addiction mechanism. After the formation of the cyclic intermediate, the Schiff base breaks down and the free carbonyl group can react with the hydroxylic group in γ-position forming a hemiacetal intermediate (Figure 4.2-c, d) [25].

Between all the possible adducts, the most detected in *in vitro* reaction is the carnosine-HNE Michael adduct.

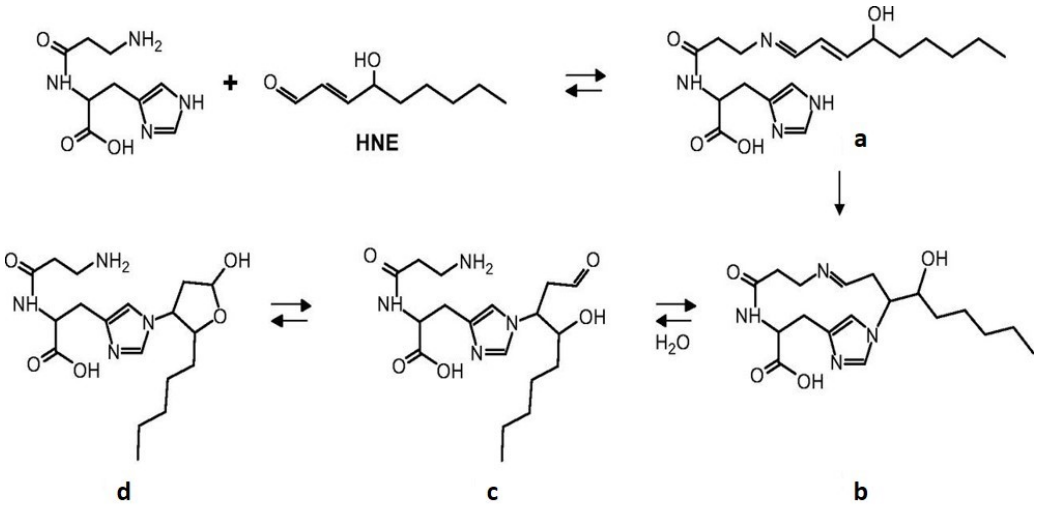

Figure 4.2*: mechanism of reactivity of carnosine with HNE. Edited from [25]*

On the contrary acrolein present a higher reactivity due to its small structure. As reported in figure 4.3, acrolein can behave as HNE via the formation of carnosine-acrolein Michael adduct (also known as carnosine-propanal) (figure 4.3-c). Nevertheless, on the primary amine of carnosine, acrolein can either form a Schiff base or a Michael adduct. Due to the small steric hindrance of acrolein, further modification of the primary amine can occur leading to the formation of two cyclization products: carnosine-(3-methylpyridinium) (figure 4.3-b) and carnosine-(3-formyl-3,4-dehydropiperidino) adducts (figure 4.3-a) [12]. Alike carnosine, carnosinol can covalently react with HNE and Acrolein.

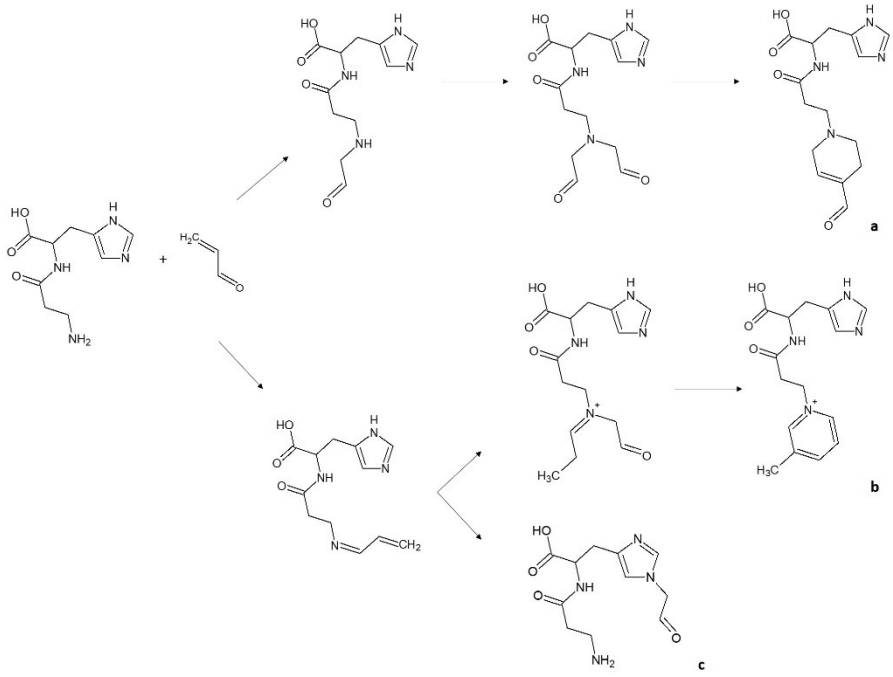

Figure 4.3*: mechanism of reactivity of carnosine with acrolein. a) carnosine-(3-formyl-3,4-dehydropiperidino); b) carnosine-(3-methylpyridinium); c) carnosine-propanal*

Carnosinol is a derivative of carnosine developed by the University of Milan and Flamma s.p.a. (figure 4.4) [26]. It has been rationally developed to be stable to serum carnosinase activity, increasing bioavailability compared to carnosine [24].

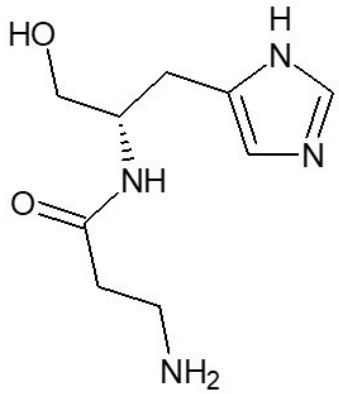

***Figure 4.4**: structure of carnosinol*

The lead optimization also improved the chemical reactivity of carnosinol against the above-mentioned major lipid-derived reactive carbonyl species (i.e. HNE and acrolein) compared to carnosine. As reported in figure 4.5 carnosinol is more reactive than carnosine against both HNE ($p<0.001$) and acrolein ($p=0.0011$, Two-Way Anova corrected Tukey). It is also to note that acrolein presents overall a higher reactivity compared to HNE.

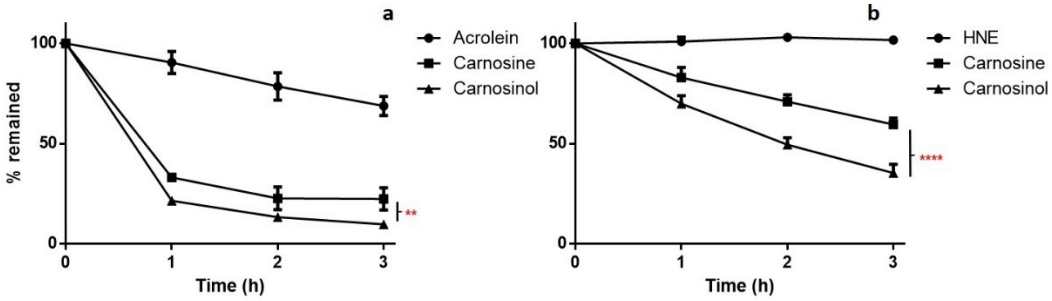

***Figure 4.5**: carnosine and carnosinol reactivity with a) acrolein and b) HNE*

To evaluate the tissue distribution of carnosinol liver, kidney, hearth, gastrocnemius, and adipose tissue were analyzed to quantify its concentration.

The tissue distribution of carnosinol in the animal models was performed using the HILIC-ESI-MRM method developed and reported in chapter 3.2.2

As it is possible to see in figure 4.6 carnosinol was detected only in animals where carnosinol was administrated. In all tissues carnosinol was detected, particularly: kidney, liver, and heart. It also seems like that the concentration of carnosinol is lower in GPx4 +/- mice compared to wt in all tissue excepted adipose tissue. The lower concentration in the genetic modify mice can be explained by the fact that these animals, when fed with HFHS diet have higher oxidative stress compared to wt mice and higher production of lipid-derived RCS (figure 4.1). Since more RCS are present in GPx4+/- mice, more carnosinol can react with them and this can be translated in a lower concentration of free carnosinol in tissues.

Moreover, based on the tissue distribution, it is possible to speculate that carnosinol can have beneficial effects epically in kidney, liver, and heart.

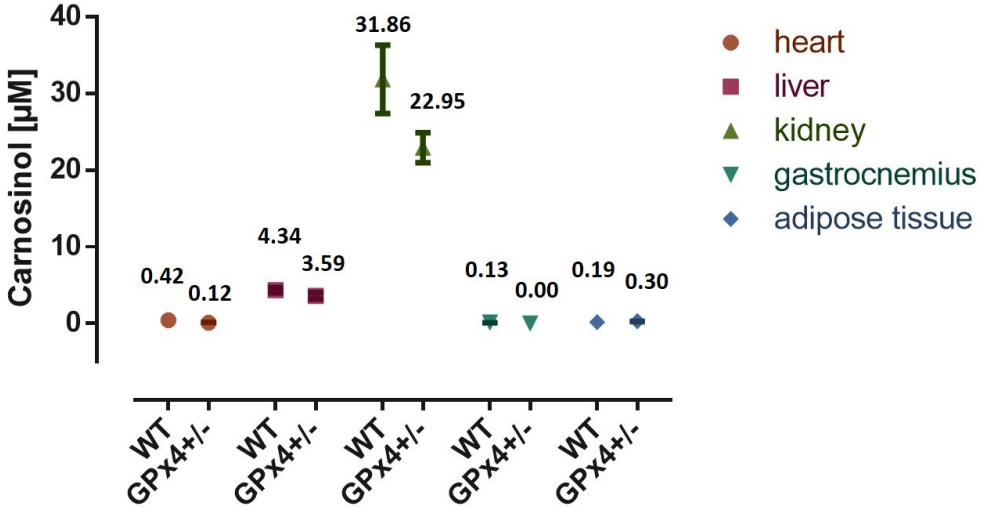

***Figure 4.6**: Carnosinol concentration in mice tissues*

The tissue with the highest concentration of carnosinol detected in both wt and GPx4+/- mice is Kidney. This data can support the results of the study of Iacobini and colleague [27], where they determined that carnosinol is able to prevent the onset and reduce the progression of diabetic nephropathy in db/db mice. The prevention/regression effect was associated with a reduce RCS-modify protein too. Nevertheless, in that paper, a tissue quantification of carnosinol is not reported. The fact that carnosinol mainly accumulate in kidney, indicates that it can be usefully targeted-develop for kidney-related diseases.

In the liver, although the concentration of carnosinol is 10-fold lower than kidneys, its activity is well established from a pharmacological point of view (figure 4.7). Carnosinol reduce the expression of pro-inflammatory proteins such as TNF-alpha, IL-6 and RAGE. Moreover, a shift from predominantly macro- to microvesicular steatosis was observed with carnosinol treatment in obese WT and GPx4+/– mice (Figure 4.7-b). Carnosinol also blunted fibrosis in the liver (Figure 4.7-c). To determine whether a HFHS diet and/or carnosinol treatment alters collagen cross-linking in the tissue, the ratio of insoluble to soluble hydroxyproline quantity in liver extracts. A HFHS diet led to an increase in the insoluble hydroxyproline fraction in WT and GPx4+/– livers (Figure 4.7-d), and carnosinol normalized these levels. Indeed, carnosinol treatment was associated with significantly lower levels of collagen overall, including soluble hydroxyproline content (Figure 4.7-e), regardless of diet or genotype. This decrease in collagen content caused by carnosinol treatment in obese WT and GPx4+/– mice does not appear to be due to decreased collagen gene expression, as we detected no effect of carnosinol on collagen-1 mRNA levels (Figure 4.7-f) [24].

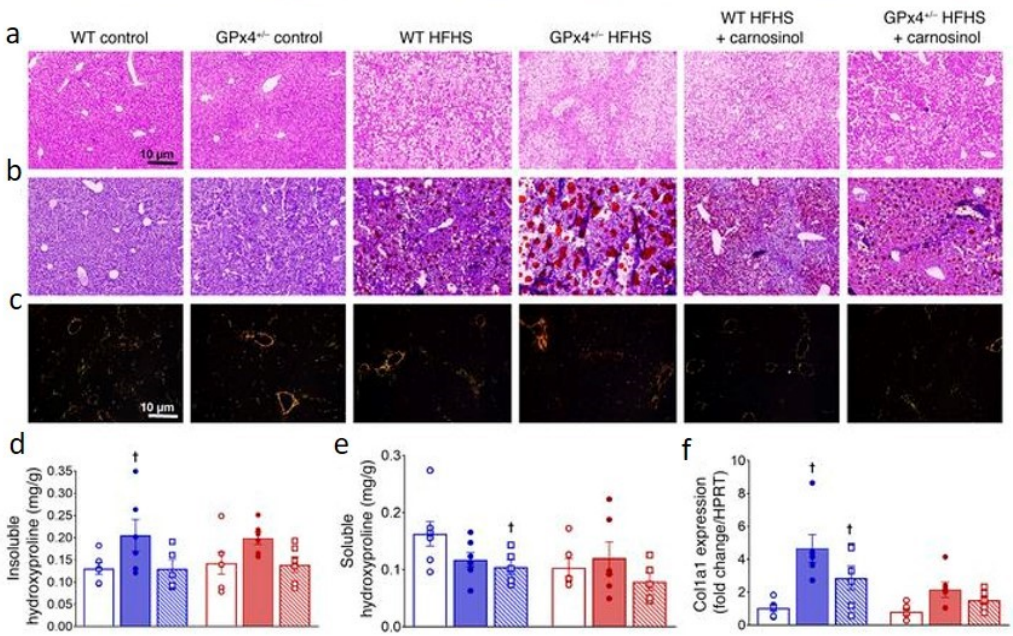

Figure 4.7*: Representative images of liver histology showing H&E staining (a), oil red O staining of triglycerides (b), and Picrosirius red staining under polarized light for collagen/fibrosis (c) in mice from each treatment group. Original magnification, ×100; scale bars: 10 µm. Both insoluble (d) and soluble (e) forms of liver hydroxyproline were quantified, along with expression of collagen 1a1 (Col1a1), determined by qRT-PCR (f). Quantified data are shown as the mean ± SEM (n = 6/group). † P < 0.01 versus control diet for each respective genotype. A 2-way ANOVA followed by Tukey's multiple comparisons test was used to test for the main effect of treatment within each genotype. Edited from Anderson et al [24]*

In muscle (i.e. gastrocnemius) even if carnosinol level were near the limit of detection or not detected, its pharmacological effect was also observed (figure 4.1-a, b). Carnosinol decrease protein-HNE adduct in gastrocnemius muscles. The decrease is higher for GPx4+/- mice than wt. It is possible speculate that for this reason carnosinol is not detected in gastrocnemius tissue of GPx4+/- mice rather than wt, since more of it has been reacted with HNE.

6.3.2. Detection and identification of carnosinol-acrolein adduct in liver

Besides determining the distribution of carnosinol, all tissues were analyzed with the nanoLC-HRMS method reported in chapter 3.2.2 for the identification of any adduct of carnosinol with RCS. Above all tissues analyzed we were able to detected a peak corresponding to the adduct carnosinol-acrolein ($[M+H]^+$ = 269.160817 m/z) in the liver matrix. The peak is found in both wt and GPx4+/- mice treated with carnosinol (figure 4.8-c, d) and it is not present in all other animal groups (figure 4.8-a, b). The chromatogram is extracted with an accuracy of 5 ppm and the retention time of the peak is equal to the retention time of the standard carnosinol-acrolein adduct synthetized (chapter 3.2.1) and added to a blank liver matrix. The final concentration of the standard adduct is 500 nM, therefore the carnosinol-acrolein adduct detected in animals is at a concentration lower than 500 nM.

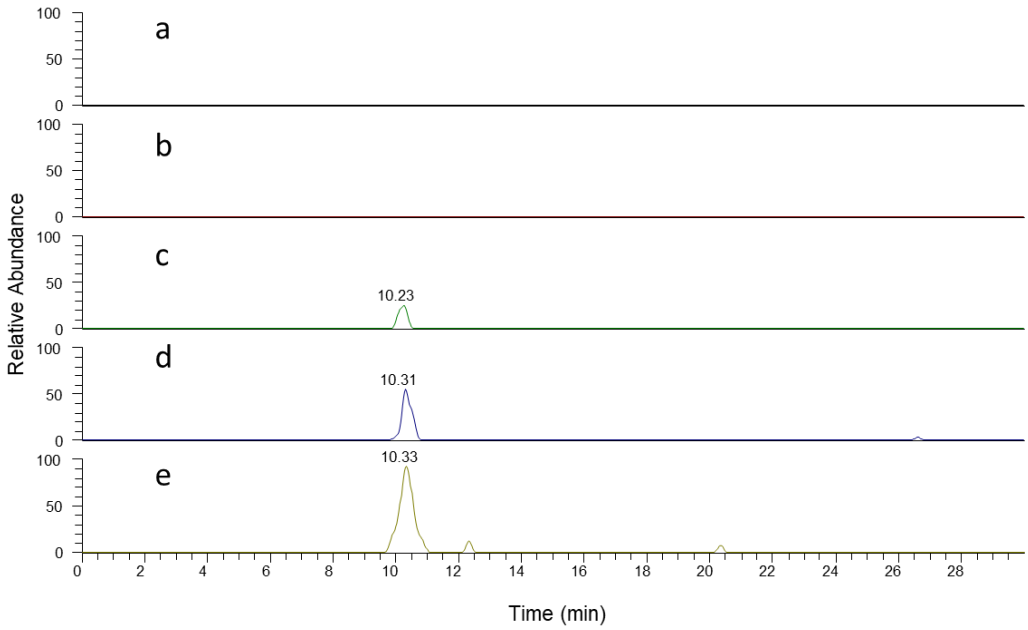

***Figure 4.8**: Extracted ion chromatogram of carnosinol-acrolein adduct (m/z 269.16082 ± 1 ppm) of the following pooled samples: a) WT + HFHS; b) GPx4+/- + HFHS; c) WT + HFHS + Carnosinol; d) GPx4+/- + HFHS + carnosinol; e) carnosinol-acrolein standard adduct spiked*

A further step of characterization was done to accurately identify the adduct. A fragmentation of the adduct base peak was carried out and the MS/MS spectra obtained from the mice tissue (figure 4.9-c) was compared with the MS/MS spectra of the standard by direct infusion (figure 4.9-a) and the MS/MS spectra of the standard, added to the blank liver matrix (figure 4.9-b).

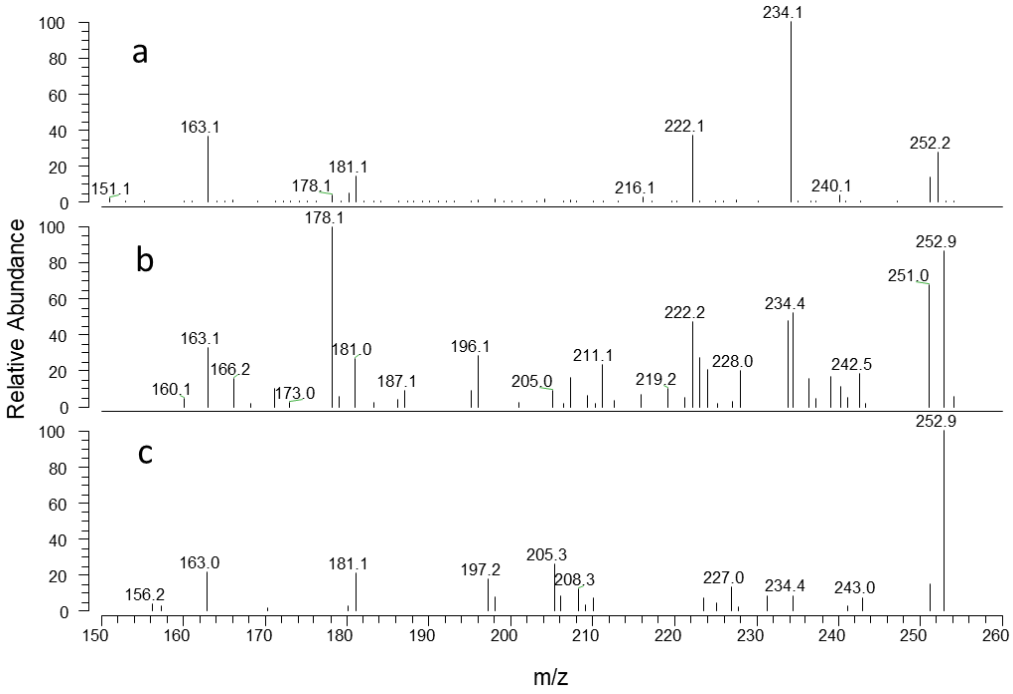

***Figure 4.9**: product ion spectra of a) carnosinol-acrolein standard in water, b) carnosinol-acrolein standard in blank liver matrix c) peak at retention time 10.31 minutes in the pooled sample GPx4+/- + HFHS + carnosinol*

From the MS/MS spectra of the standard (figure 4.9-a) by direct infusion, it is possible to identify few characteristic fragment ions (i.e. 252.2; 234.1; 222.1; 181.1; 163.1 m/z). These ions are also present in the MS/MS spectra of the standard added to the blank matrix (figure 4.9-b). However, the ion ratio between the fragments is not maintained and the spectra had a higher level of noise. This is explained by the fact that the sample is in a complex matrix.

The precursor ion (i.e. 269.16 m/z) has a low molecular weight value, and it is more likely to be accompanied with other ions with similar m/z. In the MS/MS experiment, the selection of the precursor is done with a low-resolution analyzer (linear ion trap) that is able to discriminate m/z value with an accuracy of 0.15 Da, and precursor ions for the fragmentation are filtered within a 1-2 m/z window. This decrease the selectivity of the ion selection and brings to the collision cell more than the desired ion.

The MS/MS spectra of the putative adduct in the mice tissue (figure 4.9-c) has lower noise and has the characteristic fragment ion of the carnosine-acrolein adduct, however, as the standard in the matrix, the ion ration is not maintained compared to standard outside the matrix.

Putting together the high accuracy extracted ion chromatogram (accuracy < 5 ppm), the retention time of the putative adduct comparable to the one of the standard and the presence of the characteristic fragment ions after fragmentation, it is possible to indicate the adduct identify in the liver matrix as carnosinol-acrolein adduct.

Among all tissue and all possible adducts carnosinol can form with RCS, we were able to detect only one adduct corresponding to the carnosinol-acrolein.

An explanation for the fact that no other adducts were detected is that they are not stable in biological matrices. Until now no studies on the metabolic stability of the adduct of carnosine and carnosinol with RCS have ever been reported.

Therefore, we have tested the metabolic stability of the adduct carnosinol-HNE in three matrices (figure 4.10). Carnosinol-HNE adduct is not stable through time either in kidney, in liver, or in heart. The most metabolic active tissue is liver, where after two hours of incubation carnosinol-HNE residues is of 70%.

Overall, the degradation might seem slow, but tissues were harvested from animal in fast conditions. Therefore, the adduct could have been either metabolized and/or excreted.

Unfortunately, the stability for the carnosinol-acrolein adduct could not be done since *in vitro* acrolein overreacts with carnosinol forming several by products. Therefore, the impossibility to obtain a pure product compromises the metabolic stability assay since also all other carnosine-acrolein adduct can compete with the enzyme for the degradation.

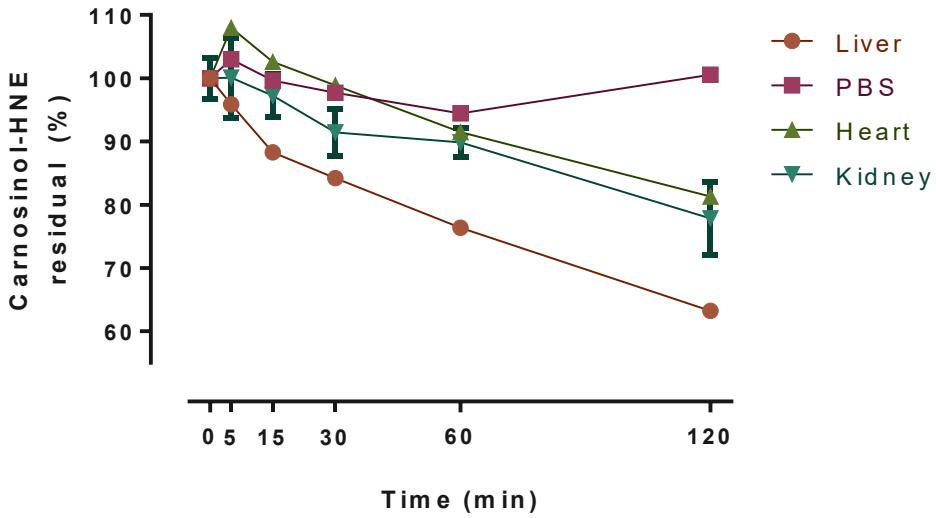

***Figure 4.10**: Carnosinol-HNE adduct stability in PBS, liver, heart, kidney homogenates at 37 °C.*

4.4. CONCLUSIONS

In this work, the HILIC-ESI-MRM method developed in chapter 3.2.2 was successfully applied and the distribution of carnosinol in animal model was determined. It is possible to speculate that there is a correlation between the relative amount of carnosinol and the two genotype and the pathology. GPx4+/- mice that have higher ROS and HNE-protein adducts level, have lower carnosinol level due to its sequestering activity of a higher level of HNE.

Carnosinol adduct with acrolein was detected and identify by means of high-resolution mass spectrometry, correspondence of retention time with a standard and partially via MS/MS analysis. In agreement with the concentration of free carnosinol, the relative quantity of the carnosinol-acrolein adduct in GPx4+/- mice was higher than in wt, indicating a higher adduct formation (Figure 4.8).

The fact that no other adducts were detected in any other tissue can be explained by the fact that the tissue harvested was done in fast condition. Considering that carnosinol-HNE adduct is not metabolically stable in biological matrices, a too long lag-time between the administration of carnosinol and the animal sacrifice could result in adduct metabolism or excretion.

Further perspectives are to obtain accurate information about carnosinol-acrolein metabolic stability. To do so the carnosinol-acrolein adduct have to be synthetized since the in vitro reaction yield to the formation of multiple adducts. Moreover, a complete characterization of the metabolites of carnosinol-RCS adducts have to be carried out to identify the last component of the metabolic pathway, therefore determining the detectable biomarker of the reactivity of histidine dipeptides with RCS.

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5 Metabolic fate of carnosine-RCS adducts

5.1. INTRODUCTION

The oxidative cleavage of lipids and sugars produces a set of conjugated ketones and aldehydes. Such compounds are often referred to as Reactive Carbonyl Species (RCS) owing to their intrinsic electrophilicity and to their chemical structure containing one or more carbonyl moieties [1]. Reactive Carbonyl Species can be classified into families based upon their structure and reactivity. Categories of RCS include dicarbonyls (e.g. glyoxal, methylglyoxal, malondialdehyde), and α,β-unsaturated carbonyls (e.g. acrolein, 4-oxononenal, 4-hydroxynonenal) [2].

Over the years the biological role of RCS has been extended to include their participation in important cellular signaling processes [3-6] whilst early studies described them as alkylants, which could trigger aberrant modifications of DNA and proteins [7, 8]. This latter process is often referred to as carbonylation and the toxicity condition following the accumulation of modified DNA or proteins in the cells is called carbonyl stress [9-11]. Correlation between carbonyl stress and the progression of some human disorders has been described for aging, diabetes, obesity, chronical renal failure, neurodegeneration, and cardiovascular diseases [12-21].

Despite the scientific effort aimed at discovering the real biological role of RCS, their metabolic fate is still far from understood, especially concerning the mechanisms responsible for their disposal as well as the alteration of such mechanisms in disease progression. As demonstrated for some of the most representative RCS (e.g. acrolein and 4-hydroxy-trans-2-nonenal), phase I and phase II reactions have been reported as the main metabolic transformations contributing to their disposal and preventing their accumulation [22, 23]. In this context, a detailed review of the metabolic pathways of HNE have been recently reported, highlighting the importance of conjugation with glutathione for its elimination [22].

Besides glutathione, carnosine is another endogenous dipeptide which can conjugate the most reactive and toxic RCS (i.e. acrolein and 4-hydroxy-trans-2-nonenal) [24, 25], thus preventing protein carbonylation and potentially reducing the carbonyl stress deriving from either disease [26, 27] or high intense physical exercise [28, 29]. The mechanism of action of carnosine and the different types of conjugates with several RCS have been characterized in vitro [24, 25]. Although carnosine activity has been demonstrated in animal models of obesity [30], diabetes [31], ischemia/reperfusion [32, 33], cancer [34], neurodegeneration [35, 36] and atherosclerosis [37] only a few pilot clinical studies on humans focused on obesity [38, 39] and diabetes [40] have been carried out.

Unfortunately, most of these studies were neither focused on characterizing the metabolism of the supplemented carnosine, nor on the identification of carnosine-RCS adducts, as proof of the ability of the peptide to deactivate RCS in vivo. The fate of carnosine adducts is a neglected topic among the scientific community, although it is of paramount importance for understanding whether carnosine conjugation can efficiently deactivate RCS and contribute to their elimination. The acquisition of such data is also fundamental for validating whether carnosine and its derivatives can be used as therapeutic agents for the mitigation of carbonylation associated with disease progression.

Our knowledge of the metabolic pathways, starting with the binding of reactive carbonyl species to carnosine and followed by the disposal of the derived conjugates, will be discussed, along with some critical and still uninvestigated hypotheses (e.g. conjugation reversibility, enzyme assisted catalysis of the reactions) which deserve further and more detailed investigation.

5.2. FORMATION OF CARNOSINE RCS ADDUCTS: DIFFERENCES AND SIMILARITIES WITH GLUTATHIONE PATHWAYS

From a general consideration of the structures of glutathione and carnosine adducts with RCS, a similar metabolic fate can be hypothesized. Specifically, both glutathione and carnosine react with HNE giving the corresponding Michael adduct, which has an aldehyde/cyclic hemiacetal interconversion equilibrium owing to the removal of the trans configuration around the double bond after the Michael reaction (figure 5.1).

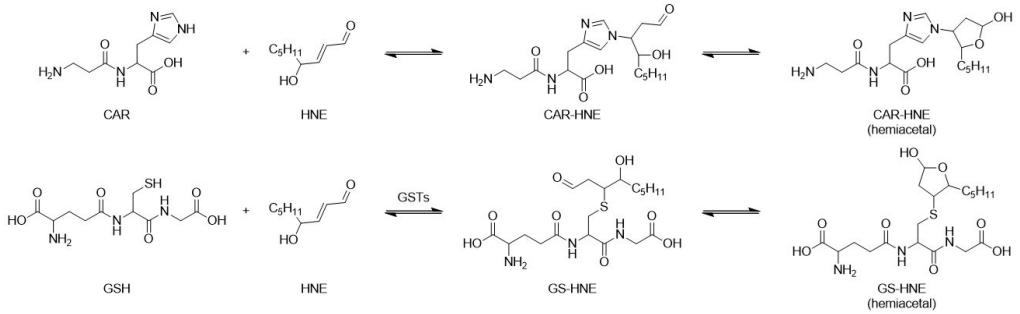

Figure 5.1*: Michael adducts generated by the reaction between 4-hydroxynonenal (HNE) and carnosine (CAR) or glutathione (GSH)*

Both reactions proceed spontaneously in vitro but some substantial differences have been observed in the reactivities of glutathione and carnosine. Specifically, the in vitro reactivity of glutathione is solely due to the nucleophilicity of its thiol moiety, its reaction rate constant being similar to that for any cysteine containing peptide (e.g. 1.21 M^{-1} s^{-1} and 1.33 M^{-1} s^{-1} for cysteamine and glutathione, respectively) and higher compared to a generic histidine containing peptide [41]. On the contrary, a self-catalytic mechanism has been reported for carnosine by demonstrating the interplay between the imidazole ring and the N-terminal amino group. Although the rate constant increase has not yet been reported, this implies that carnosine reactivity is lower than for glutathione but higher than for any generic histidine-containing peptide [24, 25].

Concerning the reactivity towards other RCS, acrolein reacts similarly to HNE with both carnosine and glutathione, although further structure rearrangements of the Michael adduct can occur (see figure 5.2) [24].

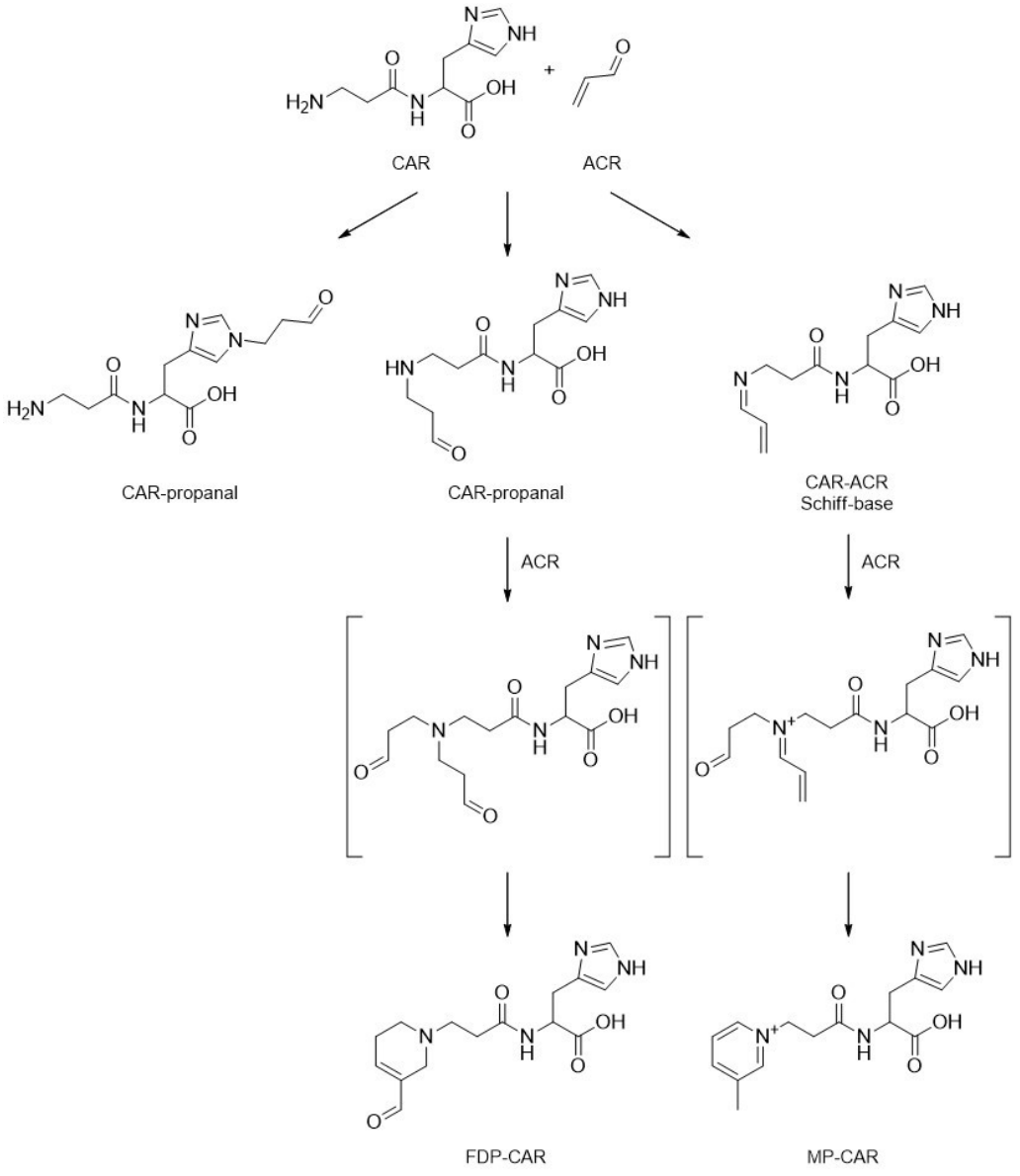

***Figure 5.2**: Carnosine reactions with acrolein to generate Michael adducts (CAR-propanal), Schiff base, N-(3-formyl-3,4-dehydropiperidino)-carnosine (FDP-CAR) and N-(3-methylpyridinium)-carnosine (MP-CAR)*

It is noteworthy that carnosine can also react with other dicarbonyls (e.g. malondialdehyde and glyoxal) with diverse reaction mechanisms, some of which also generate crosslinked structures [42]. Nevertheless, α,β-unsaturated carbonyls (e.g. acrolein, 4-oxononenal, 4-hydroxynonenal) remain the RCS class which reacts more efficiently with such a peptide.

The most relevant difference between the reactivity of carnosine and glutathione lies in the presence of a specific family of enzymes (i.e. glutathione transferases, GSTs) which catalyze the conjugation with glutathione in vivo. The role of GSTs as catalyzers of RCS conjugation has been known since the eighties [43, 44] and it has been reported that the reaction rate is increased 600-fold by this family of enzymes. By analogy, the presence of specific enzymes which enhance the reactivity of carnosine in vivo can be hypothesized, although this has not so far been investigated. However, the recent detection of carnosine-RCS adducts [45], and their correlation with some beneficial effects of carnosine supplementation [39], are somehow hard to explain. It is surprising that carnosine can exert such beneficial effects in vivo through a direct binding of RCS when, besides GST-activated glutathione, there are also other endogenous substrates (e.g. proteins) which react faster than carnosine [46]. Moreover, in view its low concentration in most cells it is unlikely that carnosine can contribute greatly to the total RCS-binding [47, 48]. The exception to this is skeletal muscle where millimolar concentrations of carnosine have been found and where the HNE binding ability of carnosine has been demonstrated [49, 50].

Another difference between carnosine and glutathione conjugation is the reversibility of the reaction. It is known that the glutathione-HNE adduct undergoes both spontaneous and GST-catalyzed retro-Michael reaction [51], while this reaction has yet to be reported for carnosine. It is notable that a higher stability to retro-Michael reaction has been reported for generic RCS adducts with the imidazole ring [52], despite a lower reactivity of such a moiety [41].

Therefore, it could be speculated that carnosine adducts can be detected in vivo because of their higher stability, and also that they are arising from the binding of HNE released from unstable adducts such as glutathione conjugates. However, no data in support of this has been published, to date.

Besides pathological conditions, also the exercise induces oxidative stress and the formation of lipid peroxidation products [53, 54]. In this field supplementation of β-alanine have been demonstrated to enhance the carnosine activity against lipid peroxidation products such as acrolein and HNE, by inducing an increase of carnosine production in muscles [28, 29]. These data are the first evidence of carnosine-RCS adducts in human tissues. Interestingly, the exercise increases carnosine level in muscles by increasing the translation of Carnosine synthase, rather than affecting carnosine absorption or transportation [28]. Besides the increase of the carnosine synthase enzyme induced by exercise, the co-administration of β-alanine (i.e. the limiting reagent of carnosine synthesis) lead to an increase in the scavenging and antioxidant capacity of the muscles during exercise [28]. These findings can explain how the body reacts to the oxidative stress during exercise, along with the evidence that exercise increases not only free glutathione and glutathione/glutathione oxidized ratio [55], but also several glutathione-related enzymes (i.e. glutathione peroxidase, glutathione reductase and glutathione S-transferase) [56-58]

5.3. CARNOSINYLATION PATHWAYS

Although carnosine could play a role in aldehyde detoxification, most of the adducts formed with RCS still present a free aldehyde moiety which is able to bind to the nucleophilic sites of proteins thus generating Schiff-base adducts. Similarly, carnosine may react with Michael adducts of proteins bearing carbonyl moieties (i.e. carbonylated proteins). Both processes produce a covalent binding of carnosine known as protein carnosinylation.

These processes were recently evaluated by Baba et al., who investigated the binding ability of carnosine-acrolein adduct (i.e. carnosine-propanal) to nucleophilic sites of protein [45]. The analysis confirmed the presence of carnosine-propanal-protein adducts, even if the exact site of interaction was not identified. The data reported suggest that two mechanisms are possible, as reported in figure 5.3: a) carnosine-binding to protein propanal moieties or b) protein binding to carnosine propanal-adducts.

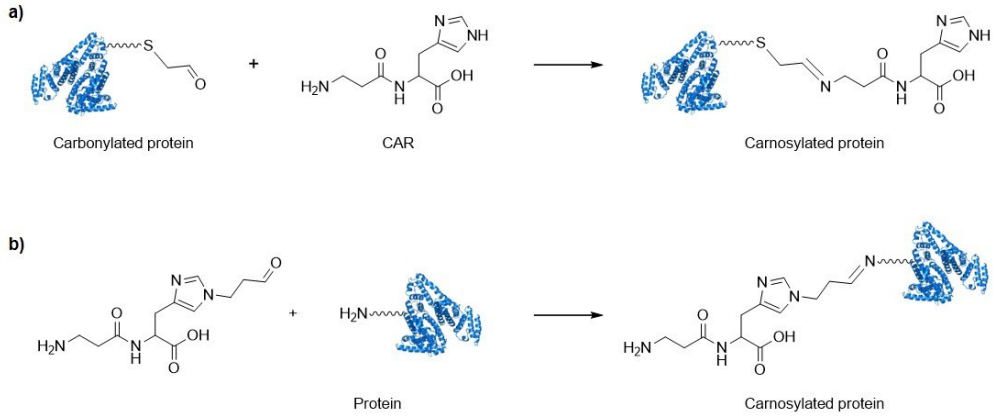

Figure 5.3*: Carnosinylation by reaction of carnosine with RCS-modified proteins (mechanism a) or by reaction of proteins with carnosine-RCS Michael adducts (mechanism b)*

These two mechanisms can lead to different considerations on carnosine activity. On one hand, the hypothesis that carnosine-RCS adducts can react with protein undergoing carnosinylation [45] can be considered the last metabolic step for RCS deactivation, although the effects of extensive protein carnosinylation and the following metabolic pathways are still unknown. On the other hand, the hypothesis that carnosine binds to carbonylated proteins could be considered important for the prevention of protein-protein cross-linking reactions [59-61].

If on the one hand carnosinylation can prevent cross-linking, on the other hand it has also been demonstrated that cysteine-propanal adducts on proteins can rearrange as reported in figure 5.4 to release cysteine residues via intramolecular cross-linking with other nucleophiles residues of the protein, ultimately leading the formation of a new Schiff-base [62].

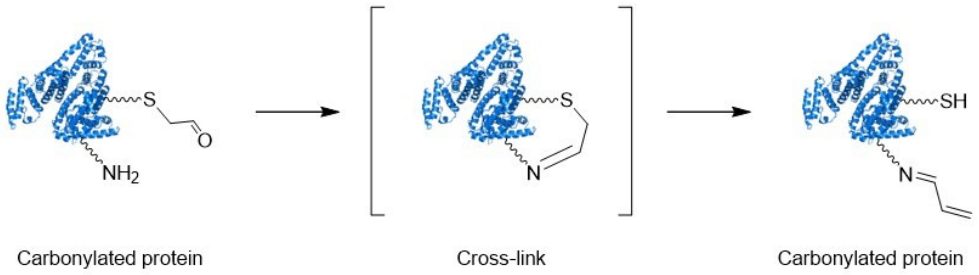

***Figure 5.4**: Intramolecular cross-linking reaction between a cysteine-propanal adduct and a proximal amino group, followed by the release of cysteine residue*

In this context, carnosine could trigger protein decarbonylation by a two-step mechanism reported in figure 5.5. Decarbonylation starts with the formation of a Schiff-base between the peptide and the cysteine-propanal adducts of proteins, followed by carnosine adduct release.

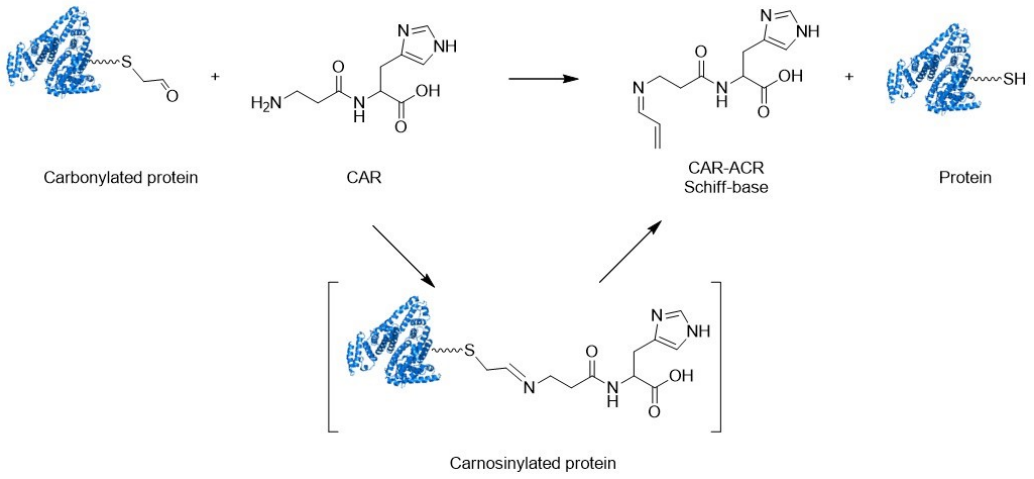

***Figure 5.5**: reaction scheme of a putative protein decarbonylation operated by carnosine*

This hypothesis, however, has still to be investigated but could represent an important mechanism explaining the reduced carbonylation of proteins observed after carnosine treatment [63-65].

5.4. METABOLIC TRANSFORMATION OF CARNOSINE-RCS ADDUCTS: DIFFERENCES AND SIMILARITIES WITH GLUTATHIONE -RCS ADDUCTS

Once generated, glutathione adducts can have two main metabolic fates, namely the reduction operated by either aldo-keto reductase (AKR) [66] or carbonyl reductase 1 (CBR1) [67] catalysis, alternatively the adducts can be oxidized by aldehyde dehydrogenase (ALDH) [68].

By analogy it is possible to hypothesize the involvement of such enzymes in the metabolism of carnosine adducts, or the presence of other enzymes exerting comparable functions. Interestingly, the reduced form of carnosine-HNE adduct (i.e. carnosine-DHN) was recently found in healthy human urine [45], confirming the presence of carnosine-HNE adduct in vivo and its metabolic transformation. Since HNE phase I metabolites (i.e. HNA and DHN) have been shown not to be reactive towards carnosine or glutathione [51], carnosine-DHN adduct is necessarily produced by the reduction of carnosine-HNE adduct, although no studies have been published concerning the enzyme responsible for this reaction.

Reduction would seem to be the preferred metabolic route of carnosine-RCS adducts, since such metabolites are the most abundant species detected for carnosine-acrolein adduct, the average concentration of the reduced species (200 pmol/mg creatinine) being threefold higher compared to the non-reduced adduct (66 pmol/mg creatinine) [45].

These findings support the hypothesis of further metabolic transformation of carnosine conjugates, once they are generated in vivo, although some differences can be observed between acrolein and HNE carnosine adducts. A seminal study recently demonstrated the in vivo reduction of carnosine acrolein adduct (i.e. carnosine-propanal) [45] by detecting carnosine-propanal, along with its reduced form (i.e. carnosine-propanol) in healthy human urine. Interestingly also HNE conjugates were detectable both as reduced and non-reduced form at concentrations lower than acrolein adducts.

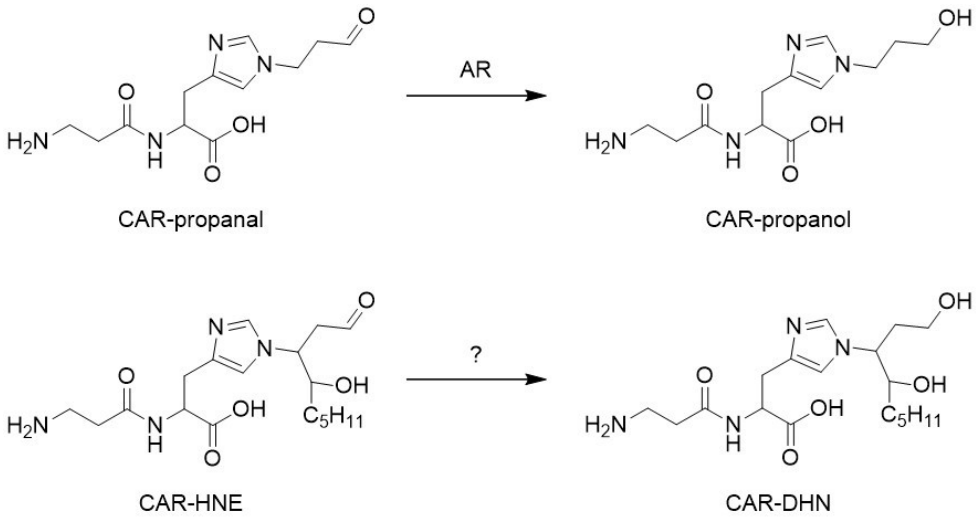

***Figure 5.6**: Reduction of carnosine acrolein Michael adduct (CAR-propanal) and carnosine 4-hydroxynonenal adduct (CAR-HNE) to CAR-propanol and carnosine-dihydroxynonane (CAR-DHN) metabolites, respectively*

The formation of the reduced form was supposed to follow the reaction scheme reported in figure 5.6 and this metabolite was the prevalent carnosine-acrolein conjugate, although propanol-mercapturic acid (i.e. a glutathione conjugation metabolite) was the most abundant acrolein metabolite confirming that GSH conjugation represents the main detoxification pathway [69].

Aldose reductase (AR, EC 1.1.1.21) has been identified as the enzyme involved in the reduction of carnosine-propanal [45]. From previous studies, AR shows to be selective for glutathionylated aldehydes, expressing higher reactivity for acrolein compared to other aldehydes (e.g. 4-hydroxyhexenal or 4-hydroxynonenal) [70]. Similarly, also carnosine-acrolein adduct has been identified as a substrate for AR, while carnosine-HNE was not reduced by the enzyme [45].

The study also evaluated the importance of AR in the detoxification carnosine-propanal. Wild type and AR-null mice tissue homogenates were used to directly evaluate the generation of the reduced form of carnosine acrolein adduct, revealing that the reduction is detectable at the first time point (2 h) and took place within six hours since no further activity was observed afterwards (up to 24 h).

The reduction of carnosine-RCS adducts can be considered an important pathway to prevent other aberrant modifications of endogenous substrates. For instance, *in vitro* experiments on BSA and a model peptide point to the importance of AR reduction in preventing protein carnosinylation [45]. The results demonstrated that AR is required for carnosine to exhibit its beneficial activity on recovery from post-ischemic reperfusion. AR-null mice showed no benefit with carnosine supplementation and carnosinylated proteins reached higher values in heart, compared to the control group (i.e. wild type animals). It was concluded that aldose reductase enhanced the activity of carnosine by reducing carnosine-propanal to carnosine-propanol preventing the post conjugation reactivity of the adducts.

A further study has revealed the presence of reduced metabolites of carnosine-acrolein conjugates in humans [39]. Obese volunteers were supplemented with carnosine (2 g/day dose) for twelve weeks and a screening for detectable amounts of carnosine conjugates was undertaken in urine and serum. Both carnosine-propanal (figure 5.7A) and its reduced form (figure 5.7B), were detectable only in urine, confirming that carnosine conjugation and reduction of the conjugate occur also in humans.

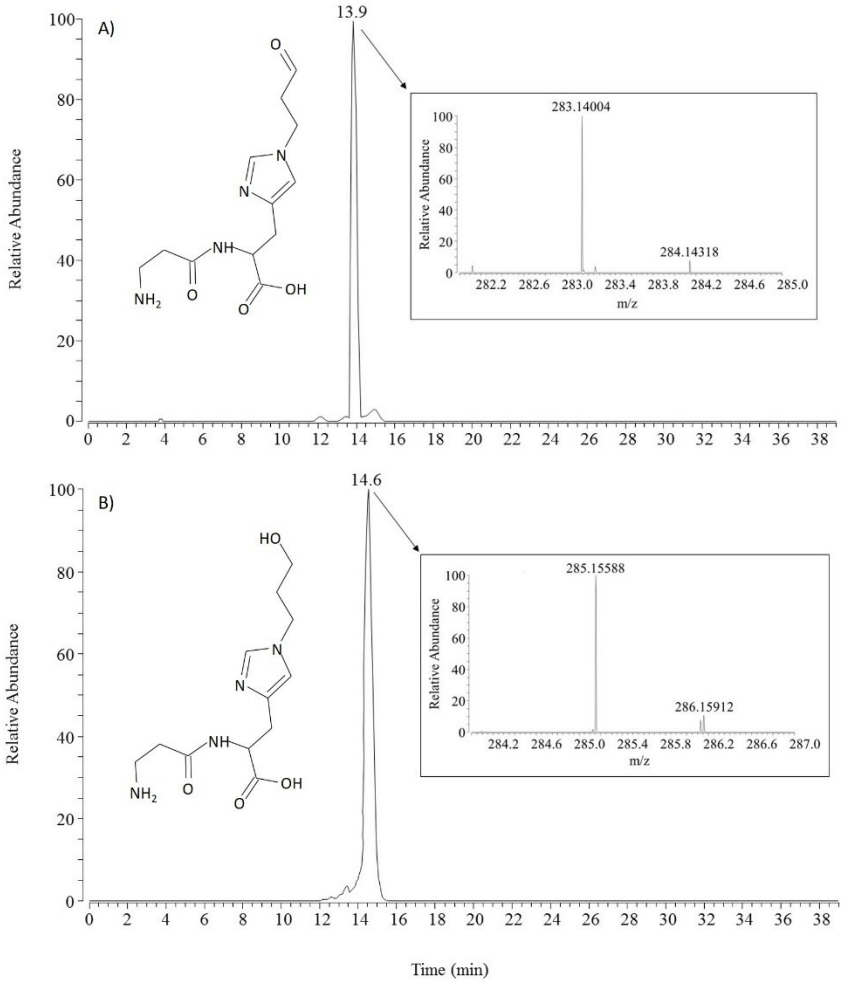

***Figure 5.7**: – identification of carnosine acrolein Michael adduct (CAR-propanal) and its reduced form (CAR-propanol) in urine of obese subjects by means of liquid chromatography-mass spectrometry (readapted from reference 38)*

It was notable that this study presented other observations on the metabolic fate of carnosine conjugates. Firstly, that such analytes were detectable also in the control group (i.e. all the subjects involved in the study before the pilot trial) and in the placebo group (i.e. subjects receiving placebo instead of carnosine for twelve weeks), and there was no variation of baseline levels during the twelve weeks of the study.

Secondly there was a linear correlation between acrolein adduct and its reduced form, indicating that the greater production of conjugates the greater the reduction. Thirdly that carnosine supplementation lead to three different response patterns, namely: I) subjects with a normal excretion of carnosine conjugates but increased urinary excretion of carnosine, II) subjects with increased excretion of both carnosine and carnosine-acrolein conjugates and III) subjects with a normal excretion of carnosine but increased excretion of carnosine-acrolein conjugates. Such diverse response could be justified by interindividual differences in terms of carnosine metabolism and acrolein basal levels. In fact, although obesity is characterized by an increase of circulating lipids, this does not necessarily imply an increase of lipid peroxidation and acrolein production. Concerning carnosinase activity, it is influenced both by genetic (i.e. CNDP1 genotype) [71] and non-genetic factors (e.g. post-translational modifications of the enzyme triggered by oxidative stress) [72] leading to fast-metabolizing and slow-metabolizing subjects, although there are no official assays for the stratification of the population phenotypes.

For subjects with a type-I response it is hypothesized that this results from a slow hydrolysis allowing carnosine accumulation, without acrolein exceeding the basal level. For subjects with a type-II response that this again results from a slow hydrolysis of carnosine but with acrolein now exceeding the basal level and requiring greater conjugation for detoxification. Finally, for subjects with a type-III response it is hypothesized that acrolein exceeded the basal level and required greater conjugation for detoxification, but as urinary excretion of carnosine was the same as for the placebo group then either the level of acrolein required the use of all the supplemented carnosine for conjugation or the carnosine was rapidly degraded by some other metabolic transformation (e.g. acetylation, hydrolysis). However, the possibility of acrolein requiring the use of all supplemented carnosine for a massive conjugation seems an unlikely hypothesis since the concentration of conjugates was not higher than with a type-II response.

Concerning the oxidative modification of carnosine adducts, no consistent data can be found in the literature about the oxidation of carnosine conjugates though a reaction scheme similar to glutathione adduct (see figure 5.8).

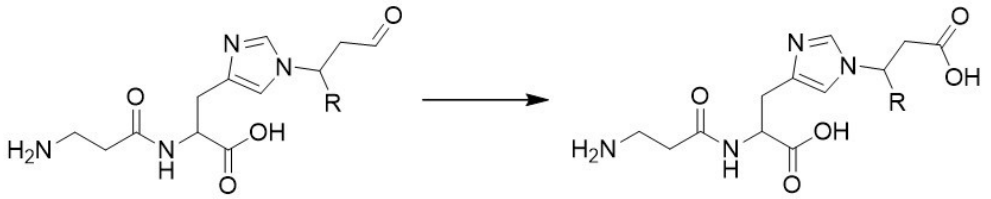

Figure 5.8*: Generation of an oxidized metabolite of a generic carnosine-RCS adduct (R= H for acrolein, $CH(OH)C_5H_{11}$ for HNE)*

The only exception is a study reporting the detection of the oxidized form of histidine-HNE [73], which was found to be increased in an animal model of obesity, compared to lean control animals. The hypothesis that such a conjugate is formed by direct binding of histidine to oxidized HNE is unlikely since, as mentioned previously, the phase I metabolites of HNE (i.e. HNA and DHN) are not reactive towards either carnosine or glutathione [51]. Therefore, there are only two possible explanation for the presence of such a histidine adduct. The first is that it is originating from oxidized carnosine-HNE after a hydrolysis, thus following a metabolic fate similar to the degradation of glutathione conjugates which are excreted as mercapturic acids (see section 5.5) [22].

The second option is that such an adduct is not derived from carnosine but from the hydrolysis of HNE conjugates with a protein or another histidine containing peptide. Either way, more investigations are required to clarify the disposal of HNE by its conjugation with histidine.

Finally, looking at other metabolic modifications of glutathione-HNE adducts such as oxidation [74] or glucuronidation [75] of the hydroxyl group of HNE after conjugation, it is unknown whether carnosine conjugates undergo a similar metabolism.

5.5. METABOLIC TRANSFORMATION OF CARNOSINE-RCS ADDUCTS: DIFFERENCES AND SIMILARITIES WITH CARNOSINE METABOLIC PATHWAYS

As reported in figure 5.9, carnosine is the substrate of several transporters belonging to the proton-coupled-oligopeptide-transporter (POTs) family. Specifically, PEPT1 [76], PEPT2 [77], PHT1 [78], PHT2 [79] have been identified as transporters for carnosine.

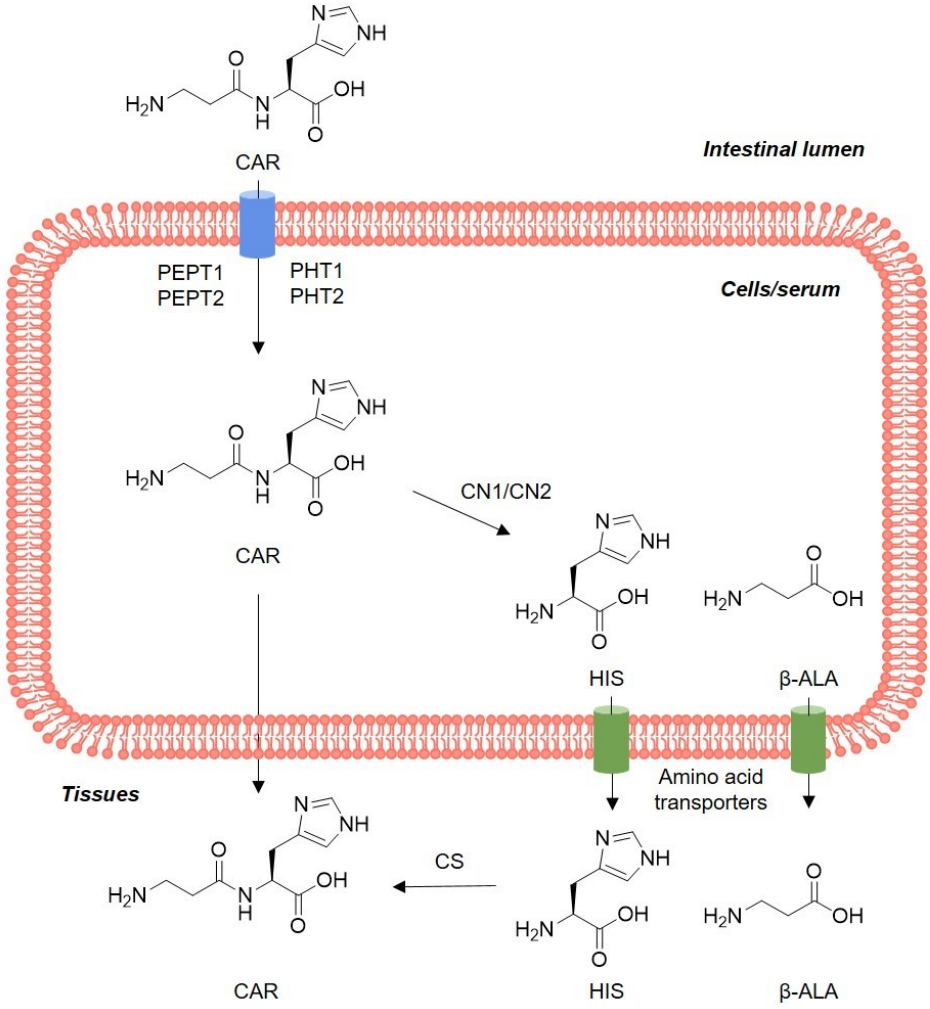

***Figure 5.9**: Carnosine absorption, hydrolysis, and re-synthesis*

Although these transporters are quite ubiquitous and they would possibly allow a distribution of carnosine in all body compartments, the peptide is not detectable in all tissues owing to the presence of two highly selective degradative enzymes (i.e. carnosinases) capable of hydrolyzing the dipeptide into its constituent amino acids (i.e. histidine and β-alanine). Two isoforms of carnosinases have been reported, namely Carnosinase 1 (EC3.4.13.20, CN1 or serum carnosinase), which is the main enzyme responsible for carnosine metabolism, and Carnosinase 2 (EC3.4.13.18, CN2 or cytosolic non-specific dipeptidase) which has a lower hydrolysis rate with an unknown role in carnosine metabolism [80].

After hydrolysis, both histidine and beta alanine can be excreted by the specific transporters LAT1 and TauT, respectively [81]. The tissue specificity of carnosine transporters is summarized in table 5.1.

Table 5.1*: Tissue specificity of the transporters involved in carnosine metabolism according to Uniprot database (https://www.uniprot.org/)*

GENE	DESCRIPTION	TISSUE SPECIFICITY
PEPT1	*solute carrier family 15 (oligopeptide transporter), member 1*	
PEPT2	*solute carrier family 15 (oligopeptide transporter), member 2*	Expressed in kidney. Not detected in intestine [82]
PHT1	*solute carrier family 15 (oligopeptide transporter), member 4*	Highly expressed in skeletal muscle. Moderately expressed in kidney, liver, and heart. Weakly expressed in colon and brain. Expressed in low levels throughout the gastrointestinal tract and in

		Caco-2 cells. Expressed in retinal fragment epithelium (RPE) and neural retina. Expressed in small intestine, stomach, duodenum, jejunum, ileum and colon [83-85]
PHT2	*solute carrier family 15 (oligopeptide transporter), member 3*	
TauT	*solute carrier family 6 (neurotransmitter transporter), member 6*	Expressed abundantly in placenta and skeletal muscle, at intermediate levels in heart, brain, lung, kidney and pancreas and at low levels in liver [86]
LAT1	*solute carrier family 7 (amino acid transporter light chain, L system), member 5*	Expressed abundantly in adult lung, liver, brain, skeletal muscle, placenta, bone marrow, testis, resting lymphocytes and monocytes, and in fetal liver. Weaker expression in thymus, cornea, retina, peripheral leukocytes, spleen, kidney, colon and lymph node. During gestation, expression in the placenta was significantly stronger at full-term than at the mid-trimester stage. Also expressed in all human

		tumor cell lines tested and in the astrocytic process of primary astrocytic gliomas. Expressed in retinal endothelial cells and in the intestinal epithelial cell line Caco-2 [87-95]

Besides the hydrolysis, other metabolic routes are known to participate in the disposal of carnosine, including the acetylation of the N-terminal amino group and the methylation of the N-π-nitrogen of the imidazole ring, although these pathways and their biological role are still not well-defined [96].

As carnosine is a natural peptide contained in foods, humans have two sources of intake for such a peptide. The first is by endogenous synthesis from its precursors (i.e. histidine and β-alanine) mediated by the enzyme carnosine synthase (EC 3.4.13.20), although this is not expressed in all tissues [97], and secondly by absorption of exogenous carnosine in meat and fish [98, 99] although this will not be relevant for vegetarians and vegans.

It is possible that the metabolism and distribution of carnosine-RCS adducts can follow similar pathways. Although there is ample evidence on the formation of Reactive Carbonyl Species in foods, the absorption of carnosine adducts has not been investigated. It has been demonstrated that acrolein can be absorbed from processed foods and eventually excreted in urine as mercapturic acid (i.e. a final metabolite of glutathione conjugation) [100, 101], whilst HNE can be produced by thermal degradation of fatty acids during cooking [102] and can be also detected in meat [103, 104]. As a result, it is possible that the formation of carnosine-RCS adducts occurs in meals and it is that these are absorbed.

Clues that RCS adducts can be distributed in multiple ways have been obtained from experiments on glutathione-HNE adducts, demonstrating that the primary route of excretion is bile and enterohepatic circulation [105]. Glutathione conjugates are also recognized by generic transporters such as human erythrocyte multispecific organic anion transporting ATPase (MOAT) and RLIP76 [106, 107]. Conversely, for carnosine adducts there is currently no evidence for passive absorption or membrane permeation, nor of any interaction with the specific transporters responsible for carnosine absorption (i.e. PEPT1, PEPT2, PHT1, PHT2). Investigations in this field should focus on whether exogenous adducts are absorbed and if endogenous adducts can move from one compartment to another.

It is notable that while bile and enterohepatic circulation are the primary routes of excretion of intact glutathione-HNE adducts, the highest concentrations of the mercapturic acid conjugates are found in urine [105]. These products are generated by the hydrolysis of glutathione adducts by gamma-glutathione-transferase (GGT) to generate cysteine adducts [108], followed by N-terminus acetylation [22].

We suggest that a similar fate can be hypothesized for carnosine conjugates since oxidized and reduced histidine-HNE derivatives (HIS-DHN and HIS-HNA) have been found in rat urine, although the corresponding carnosine conjugates were undetectable [73]. In humans, histidine conjugates with both HNE and acrolein, along with their corresponding reduced forms, have been identified in urine samples from healthy non-smokers [45]. As mentioned earlier these data demonstrate a phase I metabolism of the histidine adduct, since HNE and acrolein phase I metabolites (i.e. HNA and DHN) are not reactive towards carnosine or histidine [51, 109]. However, since histidine adducts are among the final products detectable in urine, a hydrolytic degradation of RCS adducts with histidine is necessary.

However, the origin of these metabolites is not known and may or may not involve the hydrolysis of carnosine adducts (see figure 5.10) or be generated from degradation of adducts with proteins or other histidine containing peptides. In this context it would be helpful to know whether carnosine adducts can undergo hydrolysis in vivo, either spontaneously or through the action of specific enzymes such as Carnosinase. Moreover, it would be important to investigate whether histidine adducts generated from the hydrolysis of carnosine adducts are recognized by histidine transporter LAT1.

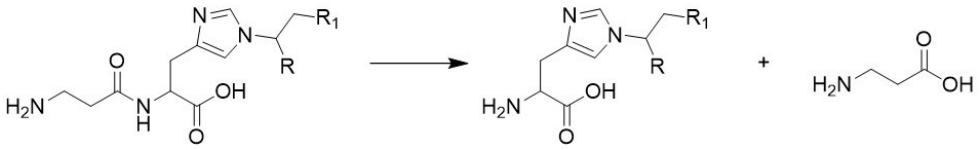

Figure 5.10*: Putative hydrolysis reaction of a generic carnosine-RCS adduct (R= H for acrolein, $CH(OH)C_5H_{11}$ for HNE; R_1= CHO for native adducts, CH_2OH for reduced metabolites, COOH for oxidized metabolites)*

Another important metabolic route of carnosine that can be shared by conjugates is the acetylation of the N-terminal amino group. The importance of this pathway is demonstrated by the evidence that N-acetylcarnosine is the most abundant carnosine metabolite in urine [98], even if the mechanism of its formation is still not completely defined and the enzyme responsible for its formation in humans is still unknown. Importantly, this pathway can be balanced by the deacetylation of N-acetylcarnosine, although this reaction has been reported only for ophthalmic preparations [110]. The metabolite (i.e. N-acetylcarnosine) has a reactivity comparable to a regular histidine peptide and lower than carnosine, since the catalytic interplay between imidazole ring and amino group is impossible after N-terminal acetylation [109]. Therefore, an activity of N-acetylcarnosine as an RCS binder in vivo is unlikely.

However, the presence of acetylated carnosine conjugates can be hypothesized since the acetylation takes place on the N-terminal group and it could possibly occur also on carnosine-RCS adducts (see figure 5.11), following a pathway that is similar to the formation of mercapturic acids from glutathione-RCS adducts [111, 112].

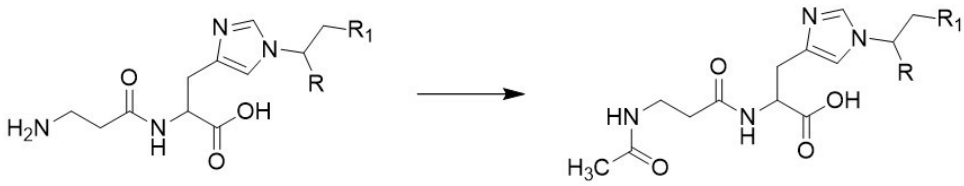

Figure 5.11*: Putative N-acetylation of a generic carnosine-RCS adduct (R= H for acrolein, $CH(OH)C_5H_{11}$ for HNE; R_1= CHO for native adducts, CH_2OH for reduced metabolites, COOH for oxidized metabolites)*

Another potential metabolic reaction involves the methylation of the N—π nitrogen of the imidazole ring. This pathway may be similar to the synthesis of anserine, which is a carnosine analog methylated on the N—π nitrogen of the imidazole ring (i.e. N—π-methycarnosine). Anserine is the most abundant histidine-containing dipeptides in mammals and poultry, except for humans and equines [96]. Anserine is a carnosine metabolite and not a product of carnosine synthase activity on N—π-methylhistidine and β-Alanine [113] since it is synthesized by carnosine methylation operated by the enzyme N-methylcarnosine transferase (EC 2.1.1.22), for which an human isoform has recently been identified [114]. Since the presence of endogenous anserine in humans has recently been demonstrated [28, 115] and human N-methylcarnosine transferase has been found in kidney together with trace levels of anserine in urine, carnosine methylation is a plausible metabolic pathway in vivo [98]. The same may be considered also for carnosine conjugates since it has been demonstrated that the N—π nitrogen of the imidazole ring is not involved in the Michael reaction with α,β-unsaturated aldehydes [116] and therefore potentially subject to methylation as reported in figure 5.12.

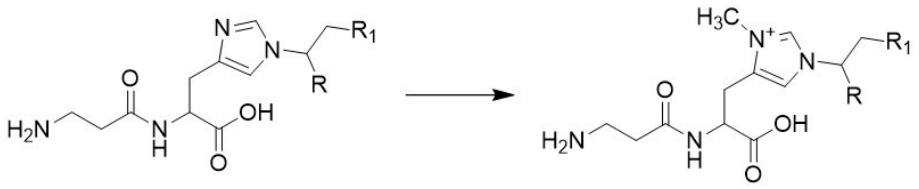

***Figure 5.12**: Putative N-methylation of a generic carnosine-RCS adduct (R= H for acrolein, $CH(OH)C_5H_{11}$ for HNE; R_1= CHO for native adducts, CH_2OH for reduced metabolites, COOH for oxidized metabolites)*

However, currently there is no evidence for the presence of metabolites of carnosine-RCS adducts generated either by acetylation of the N-terminal group or by methylation of the unadducted nitrogen on the imidazole ring, and no specific studies to address these hypotheses have been published.

5.6. CONCLUSIONS

Animal studies have demonstrated the utility of carnosine for the mitigation of carbonyl stress, whilst human and animal studies have demonstrated the potential of carnosine to slow the progression of several diseases. Despite numerous reports, in vitro and from animal models, of the activity of carnosine in binding Reactive Carbonyl Species with reduction of protein carbonylation, few such studies have been reported in humans. The main reason for this inconsistency, is that only a few studies have been committed to the identification of metabolites which can unambiguously demonstrate the mode of action of carnosine in vivo. Another reason is that human studies are more complex than animal studies since carnosine is metabolized faster in humans than rodents. Ultimately, the totally unknown fate of carnosine conjugates generated in biological matrices, if any, has also contributed to our poor understanding of the mode of action of carnosine in vivo. Further investigation is needed to characterize both the intermediates and the endpoint of the metabolism of carnosine-RCS conjugates. This is important to define the mode of action of carnosine in vivo by the correlation of the biological effects observed with the amounts of metabolites measurable in specific biospecimens (i.e. biomarkers).

To date there are no reports on the stability of carnosine-RCS conjugates, nor on their absorption/excretion processes or metabolic transformation. These topics would need to be addressed prior to any use of carnosine, or any of its derivatives, as therapeutic agents in humans.

5.7. REFERENCE

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6 Discovery of inhibitors of human serum carnosinase

6.1. INTRODUCTION

Serum carnosinase (CN1- E.C. 3.4.13.20) is the main enzyme involved in carnosine metabolism [1]. It is mainly produced in the brain released into the blood through the cerebrospinal fluid. It is also produced in a lower quantity by the liver [2]. Carnosine is rapidly hydrolyzed by CN1 as soon as it reaches the blood, and this phenomenon is the main limiting factor for the oral administration of carnosine as therapeutic. Researchers were able to induce carnosinemia (i.e. an increase of carnosine blood concentration) by an administration of a high dose of carnosine, although the plasmatic peak did not last long (i.e. 120 min) and the quantity of carnosine administrated was too high (i.e. 60 mg/kg) to hypothesize a similar therapeutic treatment [3]. Therefore, in the past years several strategies were developed to overcome this problem. The main one is the discovery and development of carnosine analogues stable to CN1 activity (e.g. D-carnosine octylester and carnosinol) [4, 5]. However, a recent alternative strategy has been investigated with the aim of developing CN1 inhibitors (e.g. carnostatine) [6].

We investigated the metabolism of carnosine to identify preferably natural compounds or commercially available drugs acting as carnosinase inhibitor. The goal is to overcome all the development processes necessary for reaching the clinical phases for a new chemical entity (as carnostatine) and accelerate the overall process. If a natural compound can inhibit serum carnosinase, it would be possible to directly test the combination of carnosine and the putative natural compound in humans.

6.2. MATERIAL AND METHODS

Chemical:

Carnosine, anserine and balenine were a gift from Flamma spa (Chignolo D'isola, BG, Italy). Carnosine hydroxamic acid was synthetized by the group of Prof. Fumagalli of the department of Pharmaceutical Sciences of the University of Milan. All other chemicals were purchased from Sigma Aldrich (Milan, Italy).

Sample preparation:

The sample preparation is as reported in chapter 3.3.1 with small adjustment. Briefly serum was diluted 1:10 in saline phosphate buffer (10 mM pH 7.4). The sera samples were preheated at 37 °C for 5 minutes. After that carnosine was added at the final concentration of 50 μM and the tested compound in a range between 50 and 500 μM. The reaction was stopped at defined time points (i.e. 0; 10 minutes) by nine volumes of acetonitrile containing the internal standard ($^{13}C_3$-D-carnosine).

LC-MS method:

The HILIC-ESI-MRM used is the one described in the chapter 3.2.2

Data analysis:

The ability of the tested molecules to inhibit the hydrolysis of carnosine in serum was identified by comparing the carnosine hydrolysis rate as measured in presence or absence of the tested compounds. Data are reported as percentage of residual serum hydrolytic activity.

6.3. RESULTS AND DISCUSSION

6.3.1. Competition of natural substrates

To understand the selectivity of CN1 on carnosine, we tested the competition activity of natural substrates such as anserine, balenine, homocarnosine and histidine by using the method we have recently developed for the measurement of serum carnosinase activity (chapter 3.3).

Co-incubating carnosine and the competitor substrate, we were able to measure the decrease of carnosine degradation. If the molecule can completely compete with carnosine hydrolysis through the time of the incubation the chromatographic peak of carnosine at the end of the incubation will be like the one at the beginning of the incubation (figure 6.1)

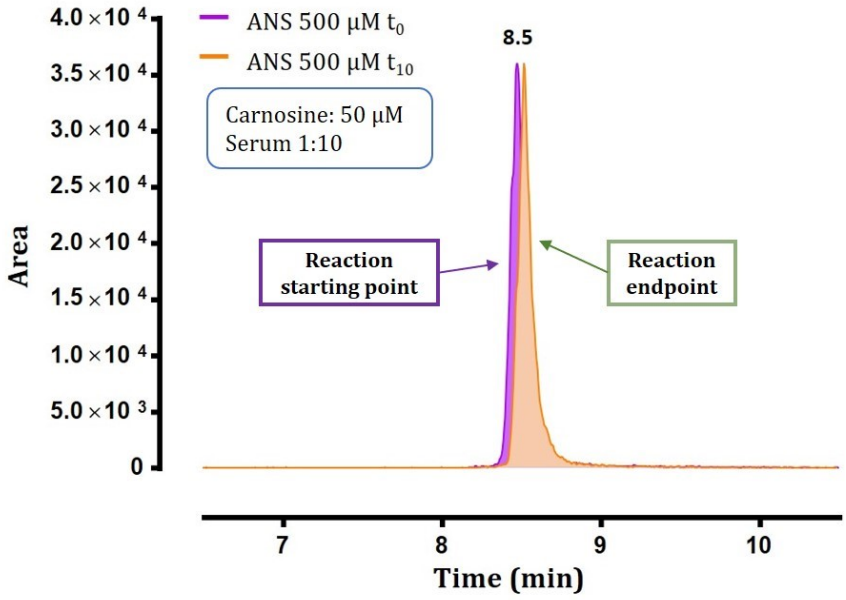

***Figure 6.1**: carnosine transition 227-110 at the beginning and the end of the incubation with 10-fold concentration of anserine.*

In table 6.1 are reported the residual serum hydrolytic activity in presence of other natural substrates competing with carnosine. It is possible to see that only anserine and homocarnosine were able to reduce nearly to 0 the hydrolytic activity at a concentration 10-fold higher than carnosine (50 µM). On the contrary, both balenine and histidine (one of the two products of the hydrolysis of carnosine) were not able to compete much with the hydrolysis of carnosine.

***Table 6.1**: residual serum activity determined for anserine, homocarnosine, balenine and histidine*

	Compound	**Residual Serum Activity %**
100 μM	Anserine	31 ± 11
500 μM		0 ± 0
100 μM	Homocarnosine	48 ± 3
500 μM		4.8 ± 1.8
100 μM	Balenine	63 ± 10
500 μM		45 ± 9
100 μM	Histidine	81 ± 5
500 μM		59 ± 11

The fact that anserine, but not balenine is able to fully compete for the hydrolysis of carnosine, is interesting since the molecules are two positional isomers. They differ only for the position of a methyl group on the imidazole ring of carnosine (figure 6.2). Anserine is methylated on the π nitrogen, while balenine on the τ nitrogen.

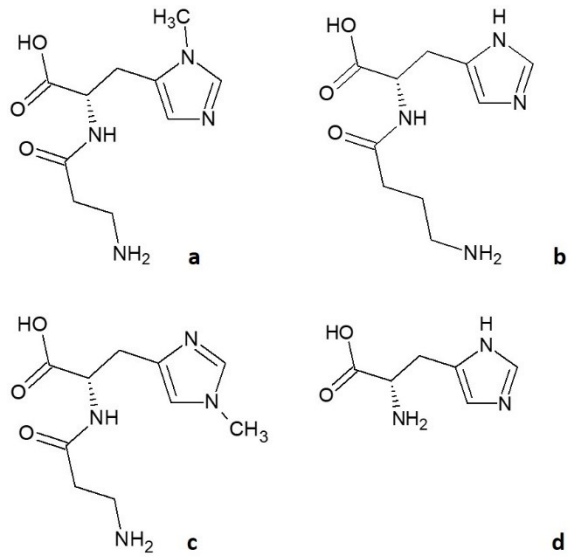

***Figure 6.2**: structure of a) anserine, b) homocarnosine, c) balenine and d) histidine*

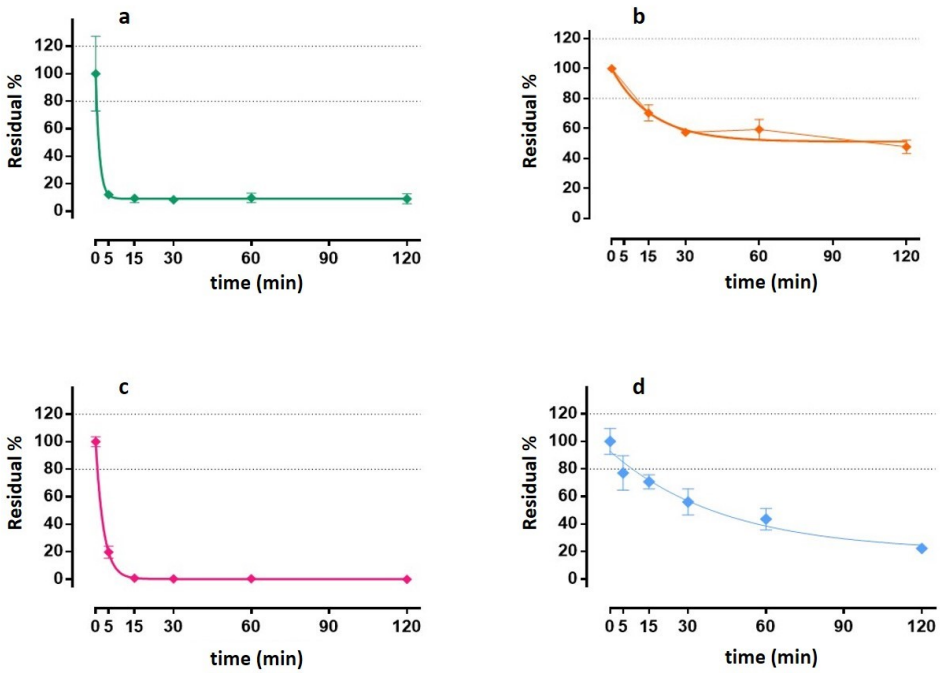

***Figure 6.3**: serum stability of a) carnosine, b) homocarnosine, c) anserine, d) balenine*

The different ability to compete with carnosine can be related to their different serum stability (figure 6.3) due to CN1 catalyzed hydrolysis (chapter 3.3). Differences that have been explained by docking and interaction energies calculation. Briefly, the methyl group of carnosine analogues changes the imidazole pose in the enzyme pocket, compared to carnosine (figure 6.4). Even though anserine loses an H-bond of the imidazole ring with Glu173 compared to carnosine, the methyl group is inserted in a hydrophobic pocket nice (figure 6.4-b). Balenine retains the H-bond of the imidazole ring with Glu173, but the imidazole ring assumes a shifted arrangement, pointing the methyl group towards a water molecule (figure 6.4-c).

Enzymatic activities are consistent with DFT-interaction energies (table 6.3). In particular the comparison of the docking results for balenine and anserine emphasizes the crucial role of the hydrophobic interactions which only anserine is able to stabilize and which can counterbalance the missed H-bond with Glu173, the role of which appears to be rather marginal compared to that of the other detected polar interactions.

***Table 6.3**: DFT-based interaction energies computed by single-point calculation of the minimized docking poses. Energies have been normalized by the total number of heavy atoms [7].*

	E(Kcal/mol)	Heavy Atoms	Enorm (Kcal/mol)
CARe	-7281.008259	16	-455.0630162
CARd	-7280.970313	16	-455.0606446
ANS	-7319.924394	17	-430.5837879
OMOe	-7206.698663	17	-423.9234507
OMOd	-7206.662257	17	-423.9213093
BAL	-7054.383112	17	-414.9637125

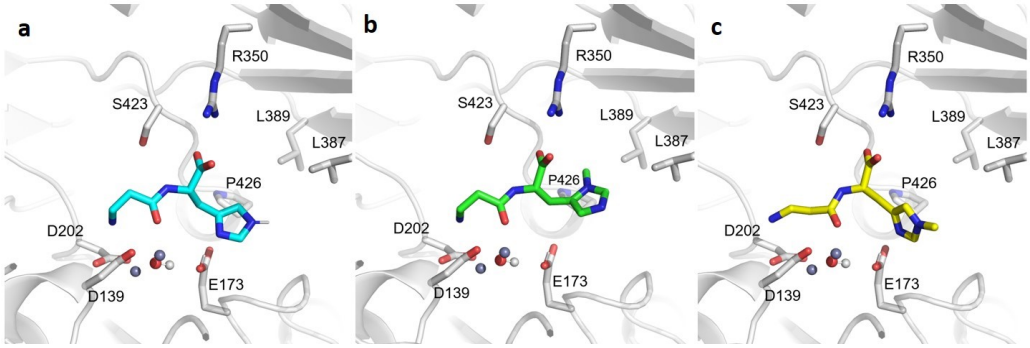

***Figure 6.4**: Comparison between the putative binding mode of a) carnosine; b) anserine and c) balenine*

Looking at this data is possible to notice that already small modification on carnosine structure can bring a great variability in term of competition with the enzyme (thus affinity).

Over the natural histidine dipeptides, carnosine is the main substrate. The methylation of the imidazole ring can bring to a slight (i.e. anserine) or a great (i.e. balenine) increase of serum stability. The addition of a methylene group to the beta-alanine chain (homocarnosine) increases the serum stability nearly as for balenine. However, homocarnosine has a higher competition activity compared to balenine.

Although few modifications were evaluated, the data give a lot of information regarding the competition between the natural substrates. These data were given to the laboratory of Prof. G. Vistoli of the University of Milan to build a virtual screening model and identify putative hits to be further tested.

6.3.2. Competition screening:

To enhance the experimental data on which build the virtual screening model for an *in silico* hit identification strategy, other compounds were tested as inhibitor of serum carnosine hydrolysis (figure 6.5).

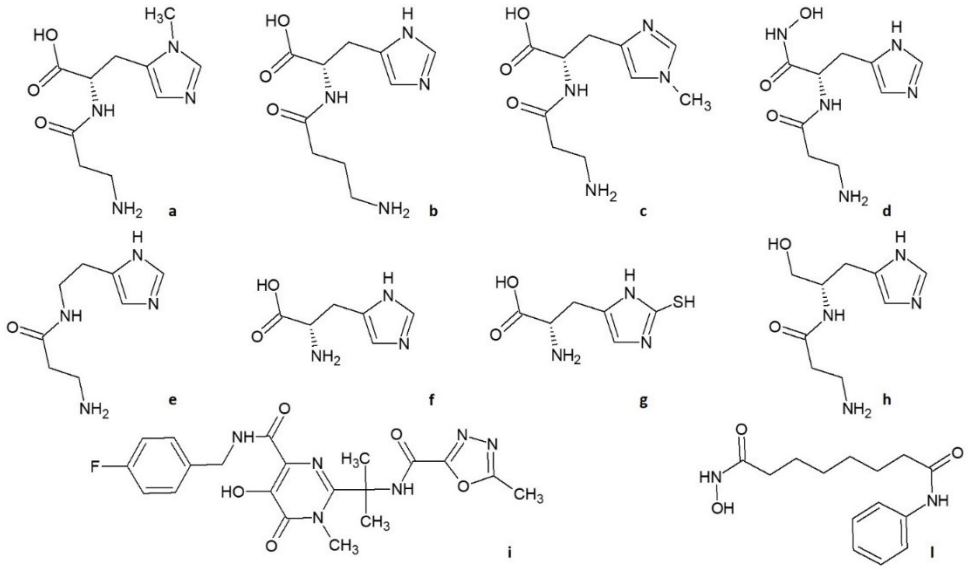

Figure 6.5*: chemical structure of the molecules screened: a) anserine; b) homocarnosine; c) balenine; d) carnosine-hydroxamic acid; e) carcinine; f) histidine; g) thiohistidine; h) carnosinol; i) raltegravir; l) SAHA*

Among these molecules there are other structural derivatives of carnosine (carcinine, carnosinol, carnosine hydroxamic acid); the product of the hydrolysis and its derivative (L-histidine, 2-mercapto-L-histidine) and two commercial drugs (raltegravir, SAHA) were tested.

CN1 is a metalloenzyme that needs two zinc ions to work. SAHA and raltegravir were tested since their mechanism of action is based on the sequestering of metal ion necessary for the activity of the target enzyme [8, 9]. On this basis carnosine-hydroxamic acid was synthetized by the group of Prof. L. Fumagalli of the University of Milan since the hydroxamic acid moiety is a known metal chelator moiety [10].

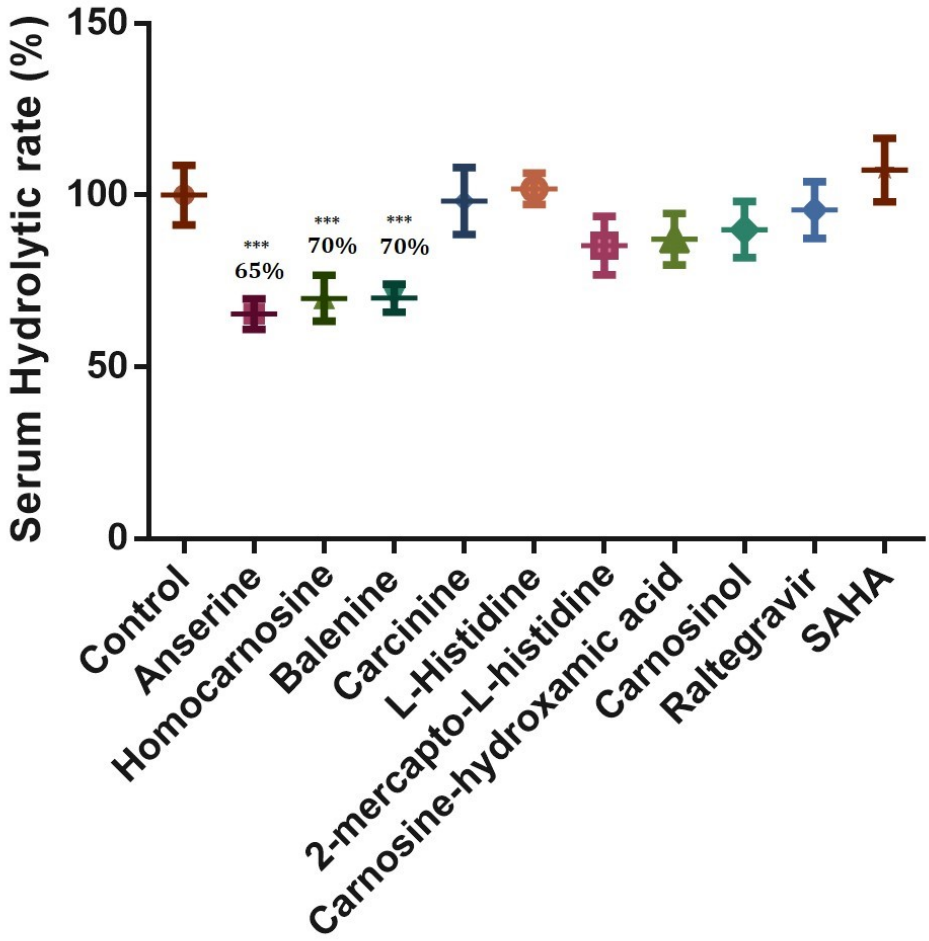

***Figure 6.6**: carnosine serum hydrolysis rate percentage of several putative inhibitors. *** = One-way Anova corrected Dunnet (p < 0.008)*

Compounds were tested in an equimolar concentration with carnosine (50 μM). As is it possible to see in figure 6.6 none of the compounds can statistically significantly reduce the carnosine serum hydrolysis. Only the natural peptides can compete and reduce the carnosine hydrolysis, to respectively 65 % (anserine) and 70% (balenine and homocarnosine) (One-way Anova corrected Dunnet (p < 0.008)).

Even if a real hit compound was not identified, IC_{50} values for anserine and homocarnosine were determined. Figure 6.7 reports the IC_{50} determination of anserine (figure 6.7-a) and homocarnosine (figure 6.7-b).

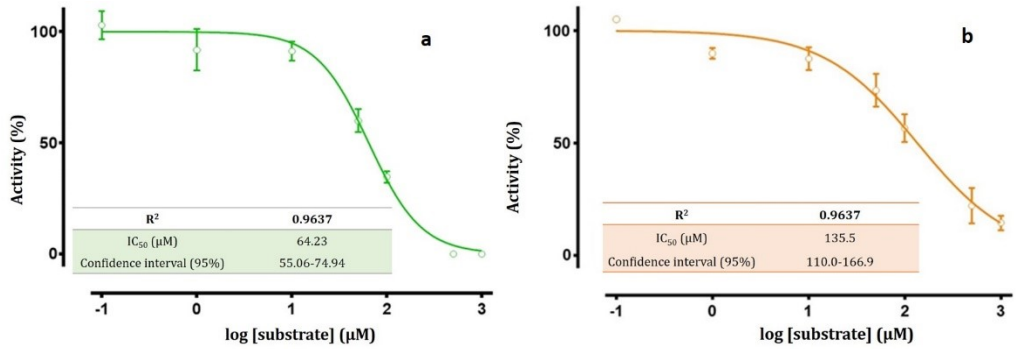

Figure 6.7*: IC50 determination of a) anserine and b) homocarnosine.*

From this preliminary data an IC_{50} corresponding to 64.23 μM and 135.5 μM were determined for anserine and homocarnosine, respectively. These preliminary results confirm the data obtained by single concentration determination (table 6.1 and figure 6.6). Anserine is a better competitor than balenine since it strongly interacts with the enzymatic pocket, but it is less stable in human serum. [7] (Chapter 3.3). Homocarnosine competition activity is higher than balenine but they have the similar stability in human serum (figure 6.3 and chapter 3.3). Balenine inhibitory activity decrease at higher concentration without being able to completely compete with carnosine degradation (table 6.1).

The fact that even small modifications of the structure of carnosine lead to drastic changes of serum stability and competition activity of derivatives, reveals useful information for further virtual screening analysis. All the results obtained were shared with the research group of Prof Vistoli (Università degli Studi di Milano) for the development of a competition model and the identification of hit compounds as inhibitors via means of docking, virtual screening and molecular dynamics experiments.

6.4. CONCLUSIONS

We have demonstrated that the direct LC-MRM method developed for the serum carnosinase activity (chapter 3.3) can be successfully applied in the screening of putative carnosinase inhibitors. We have tested in competition experiments the main natural histidine dipeptides, demonstrating that carnosine is the main substrate. Anserine and homocarnosine, if added at a tenfold concentration can completely inhibit carnosine degradation in serum, whereas balenine and histidine can only partially slow down carnosine hydrolysis.

Several other derivatives of carnosine or histidine were tested as well as two drugs known for their metal chelating properties. Nevertheless, none of the mentioned molecules were able to block carnosine degradation when incubate equimolarly with carnosine. The natural histidine peptides were the most active compounds but only a reduction of the degradation of nearly 50% was achieved.

Future works involve the screening of the candidates identified from the work of the research group of Prof. Vistoli. If one or more hits will be identified, further medicinal chemistry strategies will be carried out to develop the most active molecule.

6.5. REFERENCES

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7 Carnosine reacts with the endogenous catecholaldehyde 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL)

7.1. INTRODUCTION

Reactive carbonyl species should also include catecholaldehyde produced in the catabolism of catecholamine neurotransmitters (i.e. dopamine, epinephrine, and norepinephrine (NE)). Catecholaldehyde 3,4-Dihydroxyphenylacetaldehyde (DOPAL) is produced by both MAO-A and MAO-B metabolism of dopamine, whereas 3,4-dihydroxy-phenylglycolaldehyde (DOPEGAL) is mainly produced by MAO-A from epinephrine and norepinephrine metabolism [1]. The ability of these catecholaldehydes to react with proteins has been extensively demonstrated [1–3] and their role in the pathophysiology of neurodegenerative disorders (i.e. Alzheimer's[4] and Parkinson's[2]) and cardiac diseases [5] has been discovered. Under physiological conditions catecholaldehydes are further metabolized by ALDH and AR to generate respectively the carboxylic acid and the alcohol derivatives of the aldehyde [1]. In pathological conditions, especially during a high oxidative stress environment, several factors can determine DOPEGAL and DOPAL induced toxicity. MAO catabolism produces H_2O_2 that can further oxidize lipids forming other known lipid-derived carbonyl species (e.g. HNE) [6] that are also substrates of ALDH and AR. By competition and saturation of such detoxifying enzymes there is a global increase in the concentration of RCS, leading to protein carbonylation [1, 6]. In compartments where catecholamine neurotransmitters are highly present (e.g. dopaminergic neurons in the substantia nigra or heart tissue) DOPAL and DOPEGAL can covalently bind proteins. This reactivity is mainly due to the aldehyde moiety and the catechol ring. Catechols can undergo oxidation and generate a reactive quinone. Moreover, the formation of an oxygen radical is an intermediate in quinone oxidation [7, 8]. Carnosine was studied in vitro as a catecholaldehyde quencher, however a direct confirmation of its sequestering activity on DOPAL and DOPEGAL has not yet been published. Nelson and colleagues demonstrated the DOPAL-sequestering activity of carnosine and other antioxidant molecules (i.e. cysteine and glutathione), but the confirmation of the structure of the adduct was not reported [9].

Regarding DOPEGAL no studies have yet been reported, because a pure standard for in vitro experiments is not commercially available. Moreover, DOPEGAL is extremely unstable in aqueous environments [9]. The main way of producing DOPEGAL is via an enzymatic reaction, incubating NE or epinephrine with MAO-A [10]. According to literature bisulfite is required for the stabilization of the aldehyde formed. In fact, aldehydes react with bisulfite forming the so-called Bertagnini salt (hemithioacetal), in the same way they react with alcohol groups to form hemiacetal and acetal derivatives. The hemithioacetal derivative is stable against oxidation and it can be converted back to its corresponding aldehyde, making the salt useful for stocking DOPEGAL. Only recently an efficient chemical synthesis of DOPEGAL was described for the first time [11]. In this article the reactivity of DOPEGAL with carnosine to form a Schiff base was determined in acidic condition (0.1 M TFA solution) (i.e. the chemical condition to form Schiff bases in organic synthesis), Schiff base that further rearranges to form a stable Amadori product.

During my period as a visiting scholar at The University of Iowa, under the supervision of Prof. E.J. Anderson, I tested the reactivity of carnosine with DOPEGAL. Prof. Anderson's research is focused on cardiac disease and the role of DOPEGAL in cardiomyopathy and heart failure. In one of his recent works he demonstrated that carnosine reduces the formation of catechol-protein adduct formation after norepinephrine incubation in mitochondrial proteins of human cardiac cells [9]. Cardiac diseases characterized by heart contraction impairment [12] stimulate NE production through a positive feedback mechanism. If the increase of NE does not restore the correct contractility function of the heart, the cycle continues. MAO metabolism increases to match the increasing NE concentration and consequently generates more DOPEGAL and H_2O_2. A chronic overload of NE increases oxidative stress in cells, eventually leading to the generation of other RCS, protein carbonylation, and finally cell death [5].

The role of my research was to confirm that carnosine reacts with DOPEGAL and, if so, to characterize both the product of the reaction and the reactivity in a biological environment. Finally, carnosine-DOPEGAL could be identify as a biomarker of cardiac disease and sympathetic sensitivity.

7.2. MATERIAL AND METHODS:

Chemicals: Norepinephrine HCl, clorgiline, selegiline, TFA, TCA, ACN MS grade, water MS grade, formic acid MS grade, carnosine, sodium bisulfite, potassium phosphate were purchased from Sigma Aldrich (U.S.A.), recombinant MAO-A was purchased from Corning (U.S.A.). Trypsin 0.25% solution, DMEM, F12, and 1X PBS were purchased form Gibco (U.S.A.)

DOPEGAL Synthesis:

DOPEGAL was synthesized as reported by Nilsson, et al [10]. Briefly, norepinephrine at a final concentration of 2 mM was incubated with 2.25 μg/mL of recombinant MAO-A in potassium phosphate buffer 10 mM pH 7.4, and 5 mM sodium bisulfite for 10 hours at 30 °C, in presence of oxygen. The incubation was stopped by centrifugation at 100000 g for 30 minutes and the supernatant stored at – 80 °C prior to use.

Cell Lysate:

Experimental animals: All experiments in rodent models were conducted with approval from the Institutional Animal Care and Use Committee at the University of Iowa. C57/Bl6J wild-type (WT) mice (The Jackson Laboratory, Sacramento, CA) were used. Animals were housed in temperature and light-controlled conditions with free access to food and water.

Fibroblasts were obtained from wild type D1-D3 neonatal mice. Hearts were harvested and a commercially available kit (Pierce™ Primary Cardiomyocyte Isolation Kit, Thermo Fischer) was used to isolate cardiac fibroblasts.

Although the kit is developed for cardiomyocytes, after proprietary enzyme digestion the first cell type to adhere to the plate are fibroblasts. The isolation procedure was stopped at that step.

Cells were cultured as a monolayer in DMEM/F12 (1/1, v/v) containing 10% FBS, at 37 °C in a humidified atmosphere of 5% CO_2. At confluence cells were harvested with trypsin solution, transferred in a tube and centrifugated at 300 g for 5 minutes. The pellet obtained was re-suspended in PBS to wash the cells from residual trypsin. The washing procedure was done two times.

Cell pellets were stored at -80 °C (inducing enzyme deactivation) prior to analysis or freshly used (maintaining enzyme activity).

Cell pellets were resuspended in 300 µL of 1 mM PBS and lysed by sonication with a Sonic Dismembrator model 100 (Fisher Scientific, U.S.A.). Cell lysate was centrifugated at 10000g for 10 minutes at 4 °C, supernatant was collected, and the protein content (mg/mL) was measured with a BSA assay.

Carnosine sequestering activity:

- PBS solution: Carnosine was incubated with DOPEGAL at a final concentration of 1 mM and 100 µM respectively in PBS 10 mM pH 7.4 at 37 °C. Aliquots were sampled at 0, 1, and 2 hours and diluted 1:1 with an aqueous solution containing 1% ACN and 1% TFA to stop the reaction. Samples were directly analyzed with the HPLC-UV system or stored at -20 °C.

- Inactive cell lysate: Carnosine was incubated with DOPEGAL at a final concentration of 1 mM and 100 µM respectively in cell lysate 100 µg/mL at 37 °C. Aliquots were sampled at 0, 1 and 2 hours and deproteinized by adding TCA at a final concentration of 5% v/v. Deproteinized samples were centrifugated at 10000 g for 10 minutes at 4 °C. The supernatant was stored at -20 °C or diluted 1:5 with 0.1% FA solution prior to analysis with the HPLC-MS system.
- Recombinant MAO-A solution: Carnosine was incubated with NE at a final concentration of 770 µM and 7.70 mM respectively in phosphate buffer 10 mM at 30 °C in presence of 2.25 µg/mL of recombinant MAO-A and of oxygen. The incubation was stopped by centrifugation at 100000 g for 30 minutes. Supernatant was diluted 1:8 with an aqueous solution containing 1% ACN and 1% TFA and directly analyzed with the HPLC-UV system or stored at -20 °C. A negative control of the same reaction was done in the presence of 1 µM clorgiline (i.e. MAO-A inhibitor).
- Active cell lysate: Carnosine was incubated with NE at the final concentration of 1 mM and 50 µM respectively in active cell lysate 100 µg/mL at 37 °C. Aliquots were sampled at 0, 20, 24 hours and deproteinized by adding TCA at a final concentration of 5% v/v. Deproteinized samples were centrifugated at 10000 g for 10 minutes at 4 °C. The supernatant was diluted 1:5 with 0.1% FA solution prior to analysis with the HPLC-MS system. As negative control the same reaction was done in presence of 1 µM clorgiline and 1 µM selegiline (i.e. MAO-A and MAO-B inhibitors).

HPLC-UV analysis:

HPLC-UV analysis was done with an Agilent 1200 system with DAD detector (280 nm) (Agilent, U.S.A.). Chromatographic separation was carried out with a Phenomenex Luna column (150x1 mm, particle size, 5 µM, pore size 100 Å) with an isocratic method with a mobile phase of 97% water with 0.1% TFA and 3% ACN and a flow rate of 50 µL/min. Instrument control was done with ChemStation V.0.0.1.52 (Agilent, U.S.A.).

HPLC-ESI-QTOF:

HPLC-MS analysis was done on an Agilent 1290 infinity system coupled with an Agilent Q-TOF 5630 through a Dual ESI jet stream source (Agilent, U.S.A.). Chromatographic separation was carried out with a Zorbax column (150 x 0.5 mm; particle size 5 µm) with a gradient method (table 7.1) with mobile phase A (0.1% FA) and B (ACN 0.1% FA) and a flow rate of 15 µL/min and a 5 µL injection volume.

Ionization was carried out in positive ion mode with the following source parameters: capillary voltage 3500 V; nebulizer gas 35 psig; sheath gas 10 l/min; sheath temperature 320 °C; drying gas 5 l/min; gas temperature 300 °C; fragmentor 175 V. The analyzer was working in full mass mode with 200-700 m/z as the scan range and 1 s as the scan time. MS/MS analysis was carried out as targeted MS/MS analysis on the desired precursor ion and the desired retention time window with 100 ms as the scan time and 20 V as the collision energy. Instrument control was done with MassHunter LC/MS Data Acquisition v.B.05.01 and data visualization with MassHunter Qualitative Analysis v. B.06.00 (Agilent, U.S.A.)

***Table 7.1**: HPLC gradient method*

Time (minutes)	A (0.1% FA) %	B (ACN) %
0.00	97	3
1.00	97	3
10.00	40	60
13.00	5	95
17.00	5	95
17.01	97	3
30.00	97	3

7.3. RESULTS AND DISCUSSION

DOPEGAL was synthesized starting from NE with recombinant MAO-A in the presence of bisulfite. It is known [10] that NE has a low transformation time compared to other catecholamines (e.g., dopamine). The enzyme was saturated and let react for 10 hours. At the incubation endpoint we obtained a mixture of DOPEGAL and unreacted NE (figure 7.1).

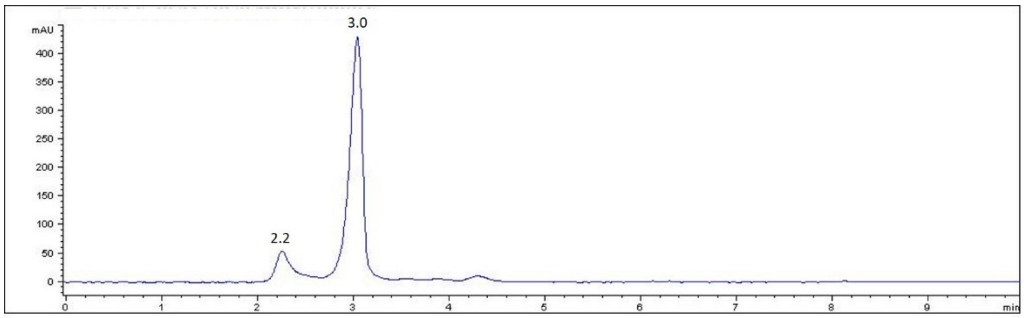

***Figure 7.1**: HPLC-UV chromatogram of DOPEGAL (RT = 2.2) produced via enzymatic transformation of norepinephrine (RT = 3.00), extracted at λ= 280 nm*

Although a low purity product was obtained, we decided to use it considering the possible interference of the residual norepinephrine. However, since the Bertagnini salt reduces the overall reactivity of the DOPEGAL aldehyde group, any kinetics consideration regarding the reactivity of the aldehyde moiety can't be done.

To test the reactivity of DOPEGAL, we incubated it with carnosine at 37 °C in phosphate buffered saline solution at pH 7.4, thus mimicking a biological environment. In figure 7.2 is possible to see the LC-UV chromatogram extracted at λ= 280 nm. From previous HPLC-UV analysis (figure 7.1) we know that the peak at 3.0 min corresponds to norepinephrine and the peak at 2.2 min is the DOPEGAL-hemithioacetal formed during the biotransformation with MAO-A. figure 7.2-a shows the initial conditions of the incubation where only NE and DOPEGAL are present as peaks. figure 7.2-b show the end of the reaction after 2 hours of incubation. A new peak at a retention time of 7.0 min is formed.

Aware of the possibility of interference of NE, the same reaction was repeated incubating carnosine with only norepinephrine in the same conditions (PBS pH 7.4, 37 °C) and no peak formation was detectable throughout all the time points (data not shown). This suggests that the peak corresponds to an interaction between carnosine and DOPEGAL.

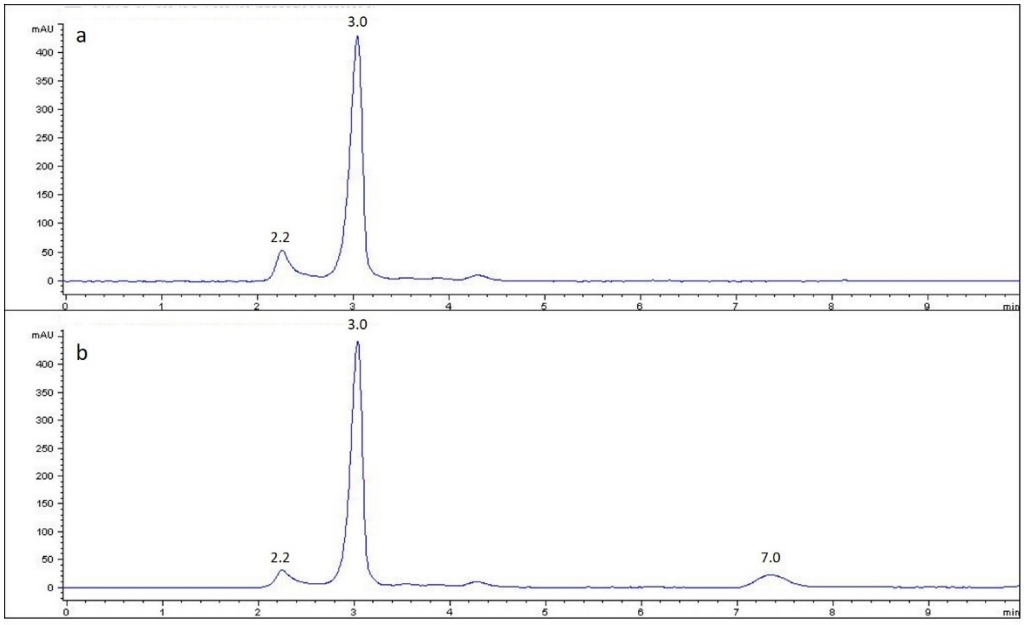

Figure 7.2*: Extracted chromatogram at λ= 280 nm: a) carnosine + DOPEGAL t = 0 min, b) carnosine + DOPEGAL t = 120 min*

To confirm that the peak corresponds to the putative adduct of carnosine with DOPEGAL we incubated carnosine in the mixture of NE and MAO-A. The incubation was done in absence of bisulfate to avoid any interference of the bisulfate salt. If NE is transformed into DOPEGAL it should react with carnosine. As a negative control we repeated the experiment in the presence of clorgiline (MAO-A inhibitor).

Figure 7.3-a shows the chromatogram of the reaction without clorgiline after 10 hours of incubation. The peak corresponding to DOPEGAL is not present, but this is expected since either it is highly unstable in the absence of bisulfate or it directly reacts with carnosine. At the retention time of 6.8 min it is possible to see a peak that could correspond to the adduct of carnosine with DOPEGAL. This speculation is supported by a parallel experiment with clorgiline (figure 7.3-b) where no peak was detectable.

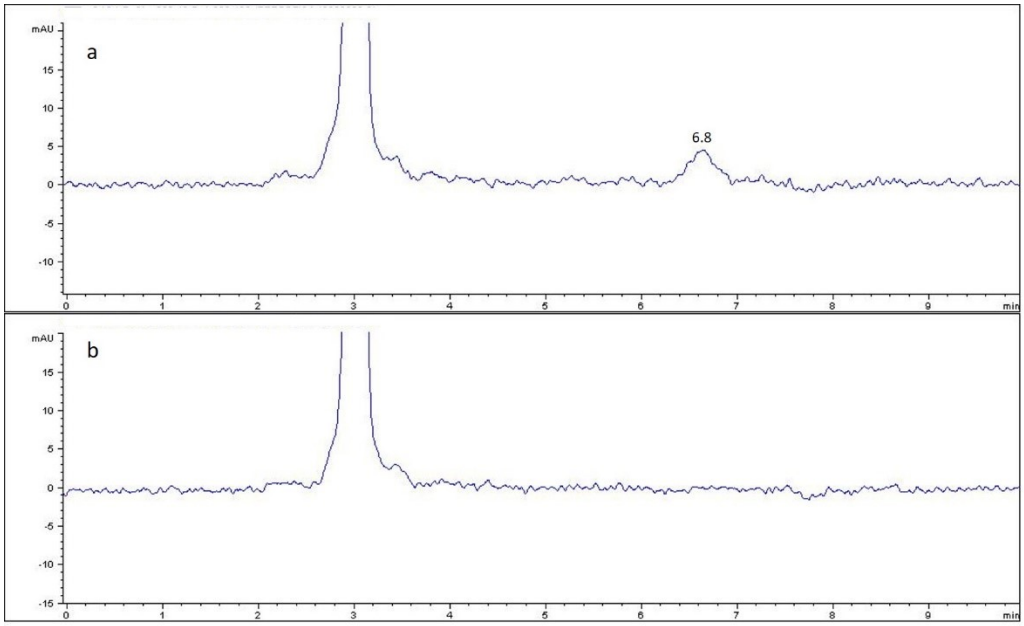

Figure 7.3*: a) carnosine + norepinephrine+ MAO-A t = 10 hours, b) carnosine + norepinephrine+ MAO-A + clorgiline, t = 10 hours*

To structurally characterize the adduct formed in the reaction between carnosine and DOPEGAL, a solution of the reaction in PBS at 37 °C was analyzed via LC-MS. Analyzing the total ion chromatogram obtained with a high resolution mass spectrometry (Q-TOF 6530) we identified only one moelcular ion corresponding to the Amadori product of carnosine with DOPEGAL. Theoretical m/z = 377.1445, experimental m/z = 377.1469, accuracy = 6.36 ppm (figure 7.4)

Although this adduct has the same mlecular weight of the carnosine-DOPEGAL Schiff base, the latter is likely not stable. Moreover, the presence of an hydroxyl group on the carbon in α to the imine moiety, the Schiff base spontaneously rearrange to the Amadori product [11]. Although carnosine-DOPEGAL adduct has been already reported in literature (room temperature, 0.1M TFA)[11] as the possible adduct between carnosine and DOPEGAL, we have for the first time demonstrated that the adduct can be formed in biological conditions (37 °C and pH 7.4). Moreover, a structural identifitcation was carried out via MS/MS - product ion experiment on the molecular ion.

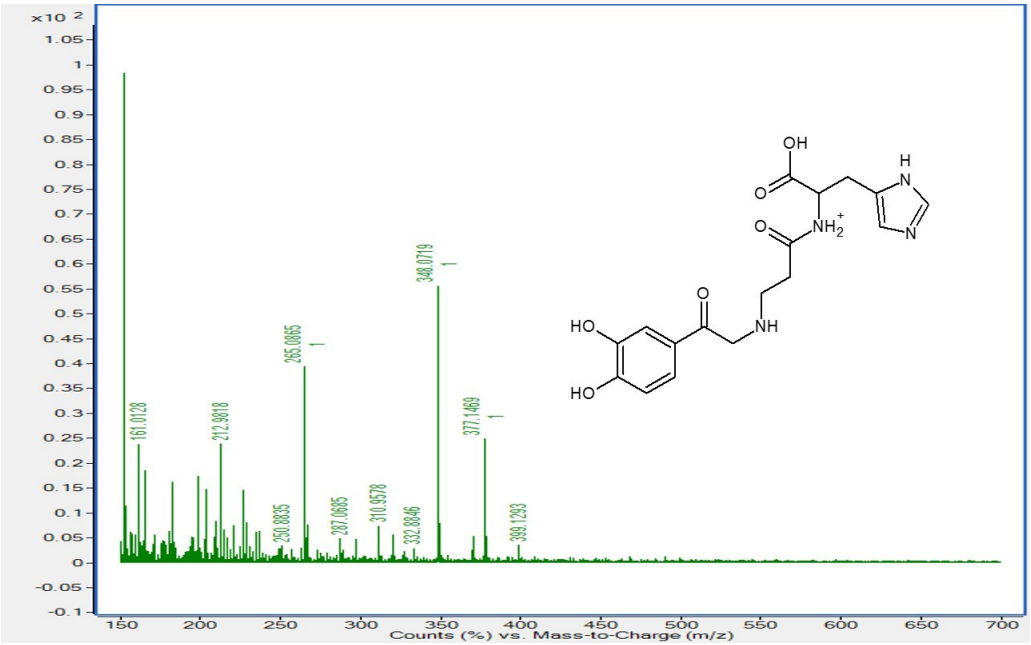

Figure 7.4*: mass spectra and carnosine-DOPEGAL adduct structure*

Figure 7.5 shows the fragmentation spectra of the molecular ion 377.14 obtained with a collision energy of 20 V and the putative fragment structures are reported for the most intense fragment ions.

The fragmentation generated characteristic fragments of carnosine like 110.07, 156.07, 164.07, and 210.1 m/z, but also generated fragments specific to the carnosine-DOPEGAL adduct such as 180.07 and 152.07 m/z.

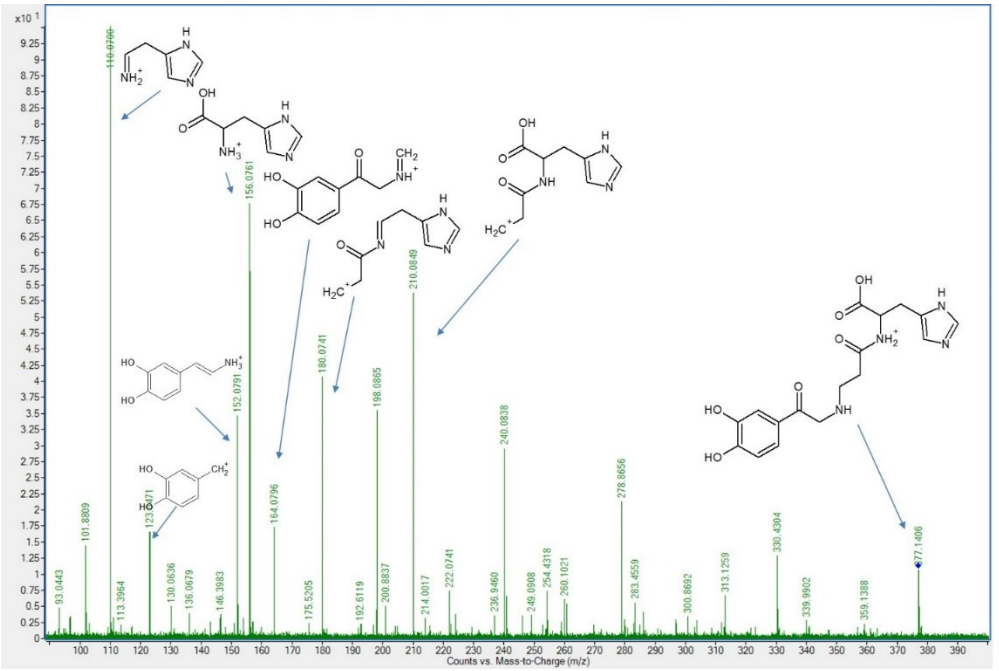

Figure 7.5*: MS/MS spectra of 377.14 m/z, CE = 20 V and fragment characterization*

Once the reactivity of carnosine with DOPEGAL was demonstrated, we wanted to do a further step to understand whether carnosine can react with DOPEGAL even in biological matrices, where other nucleophiles can compete for reaction with DOPEGAL (e.g. proteins, GSH, etc..). Carnosine (1 mM) was incubated with DOPEGAL (100 μM) in a cell lysate (100 μg/mL) at 37 °C. At defined time points, aliquots were sampled and the reaction blocked inducing protein precipitation by acidification with trichloroacetic acid (TCA 5% final concentration).

Samples were analyzed via LC-MS and figure 7.6 reports the extracted ion chromatogram of the molecular ion 377.1445 m/z ± 10 ppm. It is possible to see a time dependent increase of the peak.

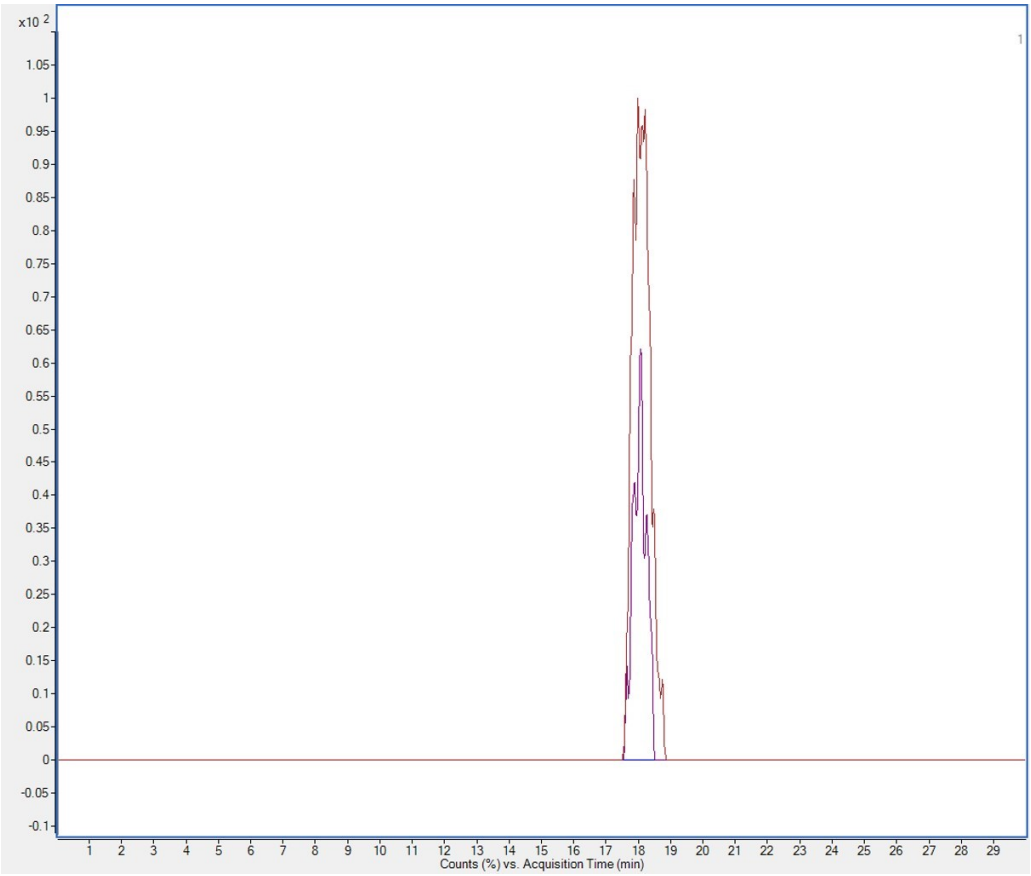

Figure 7.6*: Carnosine-DOPEGAL adduct formation in cell lysate. Blue = 0 hour; purple = 1 hour; red = 2 hours*

This experiment further confirms that carnosine can react with DOPEGAL even in a complex biological system, competing with other nucleophiles.

As the last step we repeated the experiment in an enzymatically active cell lysate (100 µg/mL), incubating norepinephine (50 µM) and carnosine (1 mM) in the presence or absence of clorgiline and selegiline (1 µM).

We were able to identify that carnosine can react with DOPEGAL formed in situ via MAO catalyzed metabolism of norepinephrine in a time dependent manner (figure 7.7-a, b, c). In the same time points of the reaction done in the presence of MAO inhibtors (i.e. clorgiline and selegiline) the adduct of carnosine with DOPEGAL was not detected (extracted ion chromatogram of molecular ion 377.1445 m/z ± 10 ppm)(figure 7.7-d, e, f).

These data are consistent with what we have found on the reaction done with the pure enzyme (figure 7.3).

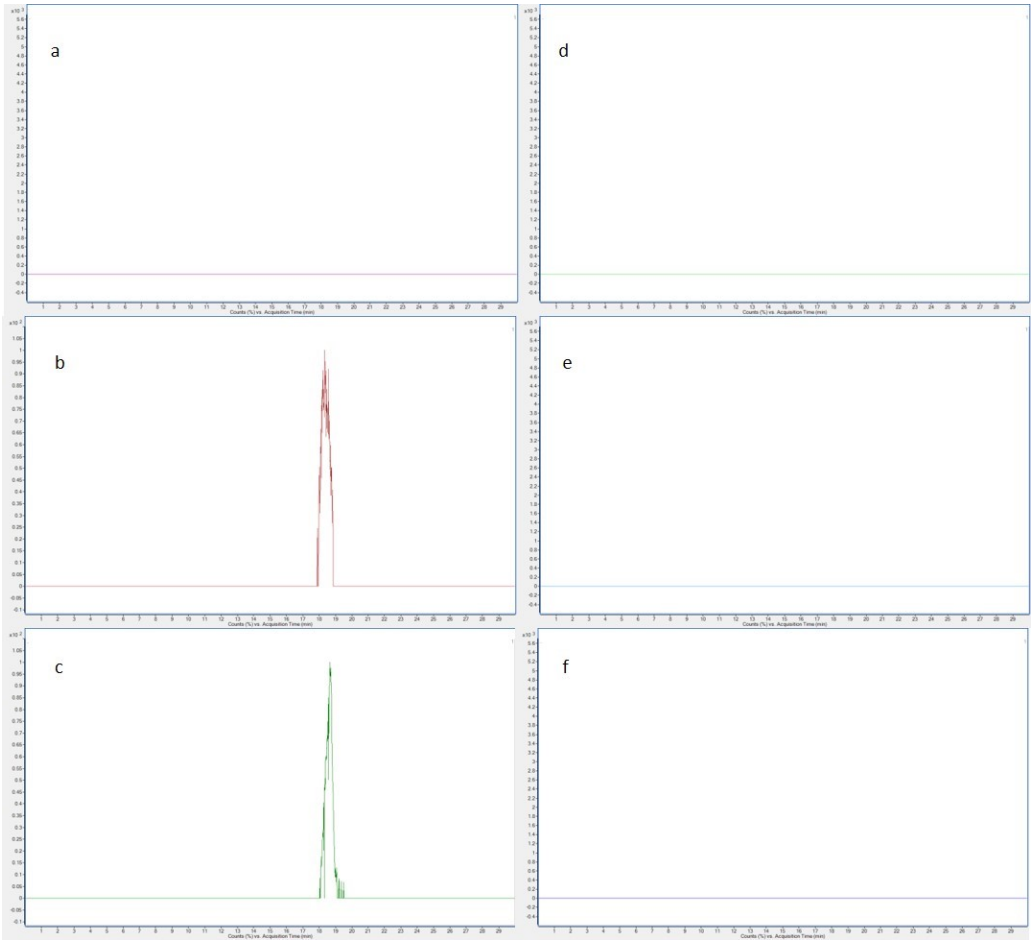

Figure 7.7*: Carnosine + norepinephrinein cell lysate a) time = 0 hours, b) time = 20 hours, c) time = 24 hours. Carnosine + norepinephrine+ MAOi, in cell lysate d) time = 0 hours, e) time = 20 hours, f) time = 24 hours*

7.4. CONCLUSIONS

With this work we have demonstrated for the first time the reaction of carnosine with DOPEGAL in a biological environment. Carnosine forms an Amadori product adduct with DOPEGAL, as confirmed via MS/MS - product ion mass spectrometry experiments. From a mechanistic point of view the quenching activity of carnosine on DOPEGAL might explain how carnosine can reduce the DOPEGAL-protein adduct as reported by Nelson and colleagues [9]. In the future, thanks to a recently published article [11] that allows to have pure DOPEGAL and DOPEGAL-carnosine standard, the reactivity of carnosine with DOPEGAL should be better characterized, especially from a kinetic point of view. Moreover, the stability of the adduct carnosine-DOPEGAL should be evaluated to understand whether it is a suitable, stable biomarker to measure catecholamine-induced tissue toxicity.

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8 HUVECs proteomics evaluation of carnosine and carnosinol treatment

8.1. INTRODUCTION

Carnosine is known to be a metal chelator, antioxidant, and RCS scavenger *in vitro*. While the *in vivo* action of carnosine is not known, several studies have been carried out that reveal interesting protein interactions with carnosine [1–3]. Novel protein interactions are of interest because the idea of a drug as a "magic bullet" has been abandoned since the discovery that molecules can induce or suppress protein expression, in addition to exerting the direct action they were designed for. Proteomics is a powerful technique that is gaining more interest in the scientific community, thanks to the never-ending development of both hardware and software technologies. Proteomics allows researchers to determine whether drug treatment yields an upregulation or downregulation of protein expression, as well as the pathways involved [4].

With the aim of evaluating the potential *in vivo* mechanisms of carnosine and carnosinol, we have started exploring this field. In literature the expression of some proteins after administration of both carnosine and carnosinol are present [1–3], but no proteomics studies have been carried out yet.

Thanks to the collaboration with the research group of Dr. Banfi of the Istituto Cardiologico Monzino, we have set up the first proteomics experiment of carnosine and carnosinol. As a starting point we decided to treat a human primary endothelial cell line (i.e. human umbilical vein cells HUVECs) with a concentration of carnosine and carnosinol that can be reached upon oral administration. As reported by a previous work done in collaboration with Prof Anderson [5], we determined the C_{max} of an oral dose of 45 mg/kg of carnosinol, which is 5 μM. Therefore, we incubated cells with carnosine or carnosinol at final concentrations of both 5 and 10 μM.

We have chosen HUVECs because such endothelial cells are vascular cells and therefore should interact with carnosine or carnosinol upon administration.

We did this first proteomics study on healthy cells without inducing any pathological conditions to evaluate:

- Whether carnosine or carnosinol induces a change of protein expression in healthy cells, and if so, which pathways are involved.
- Assuming chronic treatment with carnosine or carnosinol in an oxidative stress-based disease, what is the impact on the endothelium?

These data are important to expand upon the preventative activity of carnosine and carnosinol since some data reported in literature suggest that carnosine can prevent the onset of pathology [2, 6]. Since chronic therapy involves daily administration, we therefore expect to have an insight in the safety of the molecule on non-target healthy cells.

Carnosine is an endogenous molecule of which the physiological role has not yet been clarified. It is also studied by sports nutrition physiologists since it has an impact on sport performance. This information could also give an insight on the physiological role of carnosine and the possible benefits of supplementation inducing high carnosine plasma concentrations [7, 8].

8.2. MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Aldrich or Euroclone (Milan, Italy)

Cell Treatment

HUVECs cells were purchased from Lonza. Treatment of cells and sample preparation for proteomics and secretomics analysis was done as reported by Brisochi et al [9]. Briefly, HUVECs cells were cultured in EBM-2 medium supplemented with the EGM-2 bullet kit (complete medium) and grown at 37 °C in 5% CO_2. All the experiments were performed using cells in passages 3–6.

Treatment with carnosine or carnosinol, dissolved in medium at final concentrations of both 5 and 10 μM, was done for 24h. After that, cells were washed with medium without serum and phenol red and incubated for 16 h with serum-free medium containing carnosine/carnosinol or vehicles.

Sample preparation

Conditioned media was collected from the cells. Each sample was centrifuged for 5 min at 1000 g at 4 °C, then the supernatant was dialyzed at 4 °C using a 3500 molecular weight cut-off dialysis tube (Spectrum Laboratories, Rancho Dominguez, CA, USA) against 5 L of 1 mmol/L NH_4HCO_3 with two buffer exchanges at 4 °C for 4 h, followed by dialysis against 0.75 mmol/L NH_4HCO_3 for 4 h, 0.5 mmol/L NH_4HCO_3 for 16 h, 0.25 mmol/L NH_4HCO_3 for 4 h, and a last step against distilled water for 4 h. The samples were then concentrated by means of lyophilization and stored at −80 °C.

Protein digestion

Both the secreted protein pellets and the cell pellets were dissolved in 25 mmol/L NH4HCO3 containing 0.1% RapiGest (Waters Corporation, Milford, MA, USA), sonicated and centrifuged at 13,000 g for 10 min. The supernatants were collected and the protein concentrations determined using the Bradford method: 200 μg of each sample were heated at 80 °C for 15 min, reduced with 5 mmol/L DTT at 60 °C for 15 min, and then carbamidomethylated with 10 mmol/L iodoacetamide for 30 min at room temperature.

Digestion was performed using 4 µg of sequencing grade trypsin (Promega, Milan, Italy) overnight at 37 °C. After digestion, 2% TFA was added to hydrolyze RapiGest and inactivate the trypsin, and the solution was incubated at 37 °C for 20 min before being vortexed and centrifuged at 13,000 g for 10 minutes.

LC-MS analysis

The digested samples were further purified and concentrated by 0.2 µL C-18 resin ZipTip (Millipore, Milan, Italy) and analyzed using a Dionex UltiMate3000 nanoflow liquid chromatograph (nanoLC) that was connected in-line with an Orbitrap Fusion, equipped with a nano electrospray ionization (nanoESI) source (Thermo Fisher Scientific, Waltham, MA). The nanoLC employed an EASY-Spray PepMap C18 column (250 mm x 75 µm, particle size 3 µm, pore size 100 Å, Thermo). The autosampler compartment was maintained at 8 °C and an injection volume of 4 µL was used. Preconcentration of analytes was done on an Acclaim PepMap (50 mm x 75 µm, particle size 3 µm C18) at a flow rate of 10 µL/min with mobile phase water 0.1% TFA. After 3 minutes, the precolumn was switched online to the chromatographic column for the separation of analytes at a flow rate of 300 nL/min. Solvent A was 99.9% water/0.1% formic acid and solvent B was 80% acetonitrile/19.99 water/0.1% formic acid (v/v/v). The elution program is reported in table 8.1. Mass spectra were acquired in positive-ion mode, using the Orbitrap mass analyzer, with the following settings: spray voltage 1.8 kV, capillary temperature 275 °C, scan range m/z = 375–1500, profile mode, automatic gain control (AGC) target 4×10^5 elementary charges, maximum inject time 50 ms, number of microscans set to 1, and mass resolving power 120000 (at m/z = 400, measured at full width at half-maximum peak height).

In the data-dependent mode, the six most intense ions exceeding an intensity threshold of 2.5×10^4 counts were selected from each full-scan mass spectrum for tandem mass spectrometry (MS/MS) analysis using higher-energy collisional dissociation HCD. MS/MS spectra were acquired using orbitrap as analyzer (resolution = 30000), in centroid mode, with the following settings: isolation width at the quadrupole of 1.6 m/z units, AGC target 5×10^4 elementary charges, maximum injection time 100 ms; number of microscans set to 1; normalized collision energy 35%. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was enabled to preclude re-selection of previously analyzed precursor ions, with the following parameters: 1 repeat counts; exclusion duration 60 s; and relative exclusion mass width ±10 ppm. Charge state screening and monoisotopic precursor ion selection were enabled to exclude singly charged ions and unassigned charge states from MS/MS analysis. Each sample was analyzed in three technical replicates.

Table 8.1*: chromatographic separation gradient*

Time (min)	A (H_2O 0.1% FA) %	B (20% H_2O 0.1%FA in ACN) %
0	96	4
3	96	4
103	72	28
113	60	40
114	5	95
124	5	95
125	96	4
144	96	4

Data analysis

The instrumental raw files were analyzed by MaxQuant software v1.6.6.0 [10] set on Uniprot *Homosapiens* database by the Andromeda search engine. The quantification of peptides and related proteins was based on the LFQ intensities.

Trypsin was set as the digestive enzyme, as a fixed modification; carbamidomethylation of cysteine (+57.021 Da) as a variable modification; methionine oxidation (+15.995 Da), N-terminal acetylation (+42.011 Da), and LFQ minimum ratio count to 2 were set as further parameters. The interpretation and visualization of results from MaxQuant software were performed using Perseus software (v1.6.1.3, Max Planck Institute of Biochemistry, Germany) [11]. Statistical parameters ($p < 0.05$; adjusted p-value <0.01) were set to identify the statistically different abundant proteins between samples. Data were further analyzed with STRING (v.11.0) [12], based on Gene Ontology database and Ingenuity Pathways Analysis (last release; Qiagen)in which the statistical enrichment of involved pathways is performed by the right-tailed Fisher's exact test, in correlation with QIAGEN Knowledge Base, assigning a p-value. For graphs, python (v 3.8.2) was used via Jupyter notebook.

8.3. RESULTS

The nanoHPLC-HRMS analysis was done at the UNITECH OMICS facility of the University of Milan. A label free quantitative analysis (LFQ) was carried out. Raw data were than analyzed using MaxQuant and Perseus software for protein identification and a first statistical analysis. For the peptide spectrum match and the quantitative analysis in Perseus a false discovery rate (FDR) of 1% was applied to be as stringent as possible, increasing the reliability of the data. An FDR of 1% indicates that the proportion of falsely differentiated abundant proteins is lower than or equal to 1%. FDR is a set property, and it corrects the calculated p-value to reduce misleading results. P-values have been calculated based on three biological replicates and three technical replicates for each condition. The degree of correlation between sample replicates (biological and technical) was assessed by multi scatter plot of LFQ intensities and Pearson correlation coefficient was calculated for each condition. Coefficients of both proteomics (table 8.2) and secretomics (table 8.3) analysis were close to 1, indicating a high reproducibility of the technical and biological replicates.

***Table 8.2**: Descriptive statistics of Pearson coefficient scatter plot of LFQ intensities between technical and biological replicates in proteome analysis*

Proteome	Concentration (μM)	Mean ± SD	CV%	Min-Max
CTRL	10	**0.98596** ± 0.00465	0.47205	0.97897 - 0.99308
Carnosine		**0.98213** ± 0.01066	1.08533	0.96469 - 0.99541
Carnosinol		**0.99162** ± 0.00449	0.45292	0.98481 - 0.99700
CTRL	5	**0.98955** ± 0.00370	0.37384	0.98641 - 0.99722
Carnosine		**0.98859** ± 0.00469	0.47449	0.98166 - 0.99652
Carnosinol		**0.97400** ± 0.01182	1.21399	0.96068 - 0.99588

***Table 8.3**: Descriptive statistics of Pearson coefficient scatter plot of LFQ intensities between technical and biological replicates in secretome analysis*

Secretome	Concentration (μM)	Mean ± SD	CV%	Min-Max
CTRL	10	**0.98220** ± 0.00885	0.47205	0.96801 - 0.99621
Carnosine		**0.98153** ± 0.01136	1.08533	0.96353 - 0.99666
Carnosinol		**0.98290** ± 0.00861	0.45292	0.96859 - 0.99694
CTRL	5	**0.96669** ± 0.01861	0.37384	0.94361 - 0.99560
Carnosine		**0.97521** ± 0.01107	0.47449	0.96296 - 0.99510
Carnosinol		**0.95901** ± 0.02125	1.21399	0.93299 - 0.99576

From this first quantitative and statistical analysis for the treatment with 5 μM as a final concentration of carnosine and carnosinol, we overall identified 2819 proteins for the proteome and 1402 proteins for the secretome.

For the treatment at 10 μM as a final concentration, we overall identified 3141 proteins for the proteome and 1586 proteins for the secretome.

8.3.1. Secretome

Although we identified a high number of proteins in both the experiments, neither the treatment at 5 μM nor the treatment at 10 μM for either compound induced an increase or decrease of the expression of many proteins. From a statistical point of view (figure 8.1), in three experiments out of four there is a statistically different abundant protein expression in the treated samples compared to the control. This is because the values of the two-tailed t test for each protein are not randomly distributed throughout all p-values but there is a cluster at the lower p-values.

Only the treatment of 10 μM carnosinol did not produce a differential expression of proteins in a statistically significant way. All the observed variations were random (figure 8.1).

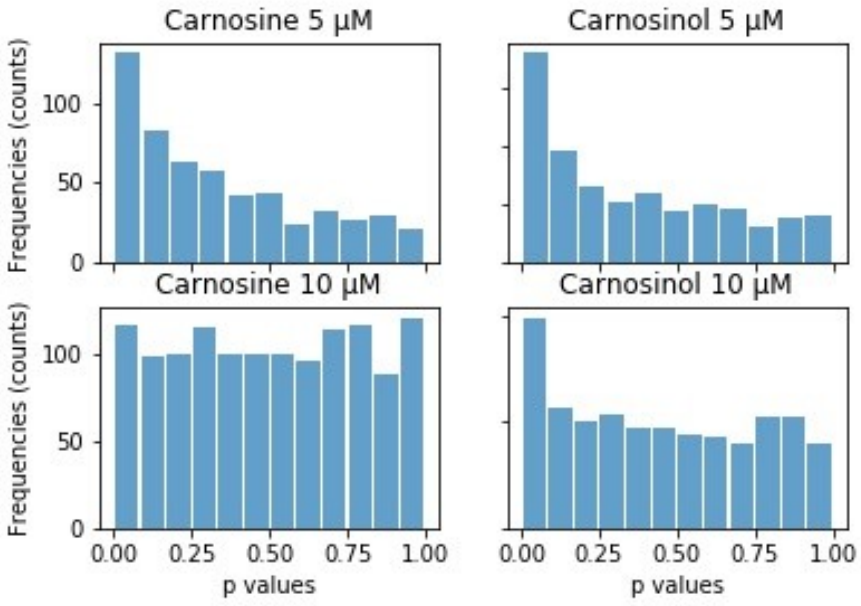

***Figure 8.1**: p-values histogram for secretome analysis*

Although this is a good sign, indicating that our experiments were carried out correctly, after further correction in order to reduce the number of false positives (Benjamini–Hochberg correction, i.e. FDR at 0.01) ,the number of statistically significant proteins decreased. Of these proteins we are interested in only those that have a fold change (up or down regulation) greater than or equal to 1.5. Considering all these parameters, only one protein per condition was identified (figure 8.2).

For the treatment with 5 μM carnosine, ICAM2 is 2.1-fold less expressed in treated cells. ICAM-2 is a protein from the intercellular adhesion proteins family which has been reported to be highly expressed in endothelial cell and may have a role in the interaction with immune system cells. Unlike ICAM-1, ICAM-2 is not upregulated after inflammatory stimuli [13].

For the treatment with 5 μM carnosinol, the only protein identified was Fibrillin-1, that compared to the control is 4.4 time less expressed. Fibrillin-1 is a component of extracellular matrix fundamental in the structural support of cells and tissue [14, 15].

For the treatment at 10 μM neither carnosine nor carnosinol induced a statistically different protein expression (figure 8.1-c, d).

From these results we can say that carnosine and carnosinol do not affect the secretome of endothelial cells in the experimental conditions tested.

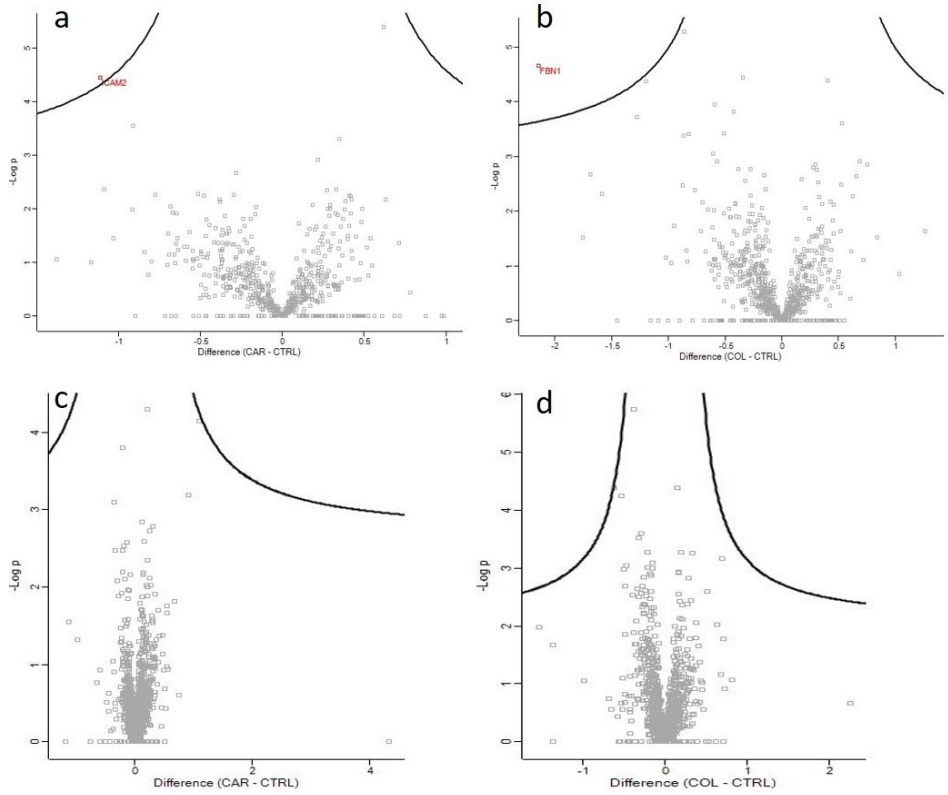

Figure 8.2*: Volcano Plot related to the secretome analysis, a) carnosine 5 μM; b) carnosinol 5 μM; c) carnosine 10 μM; d) carnosinol 10 μM*

8.3.2. Proteome

As in the secretome analysis, the histograms of the p-values calculated from a two-tailed t-test indicate a statistically different proteins expression in the treated samples compared to the control (figure 8.3). Already at a first glance we can say that treatment with 10 μM as final concentration has a bigger impact than the treatment with 5 μM, because p values are more skewed on the left side of the graph. Moreover, the treatment with carnosinol induces a higher differentiation in protein expression compared to carnosine.

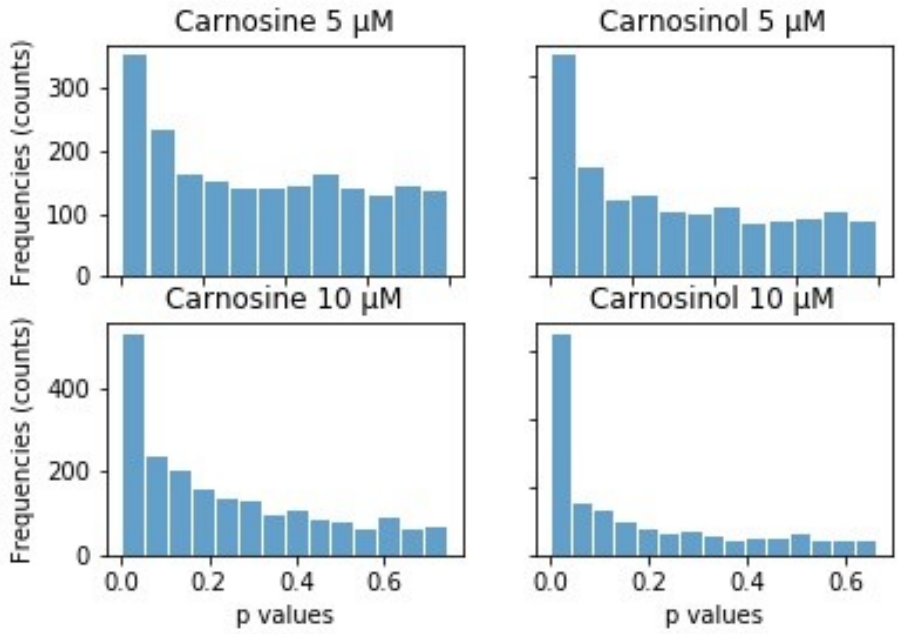

***Figure 8.3**: p-values histogram for proteome analysis*

Applying the Benjamini–Hochberg correction (i.e. FDR at 0.01) and selecting only proteins that have a fold change in the difference expression of 1.5 or greater, only the treatments with 10 µM of carnosine and carnosinol have a statistically significant number of differentiated abundant proteins (figure 8.4).

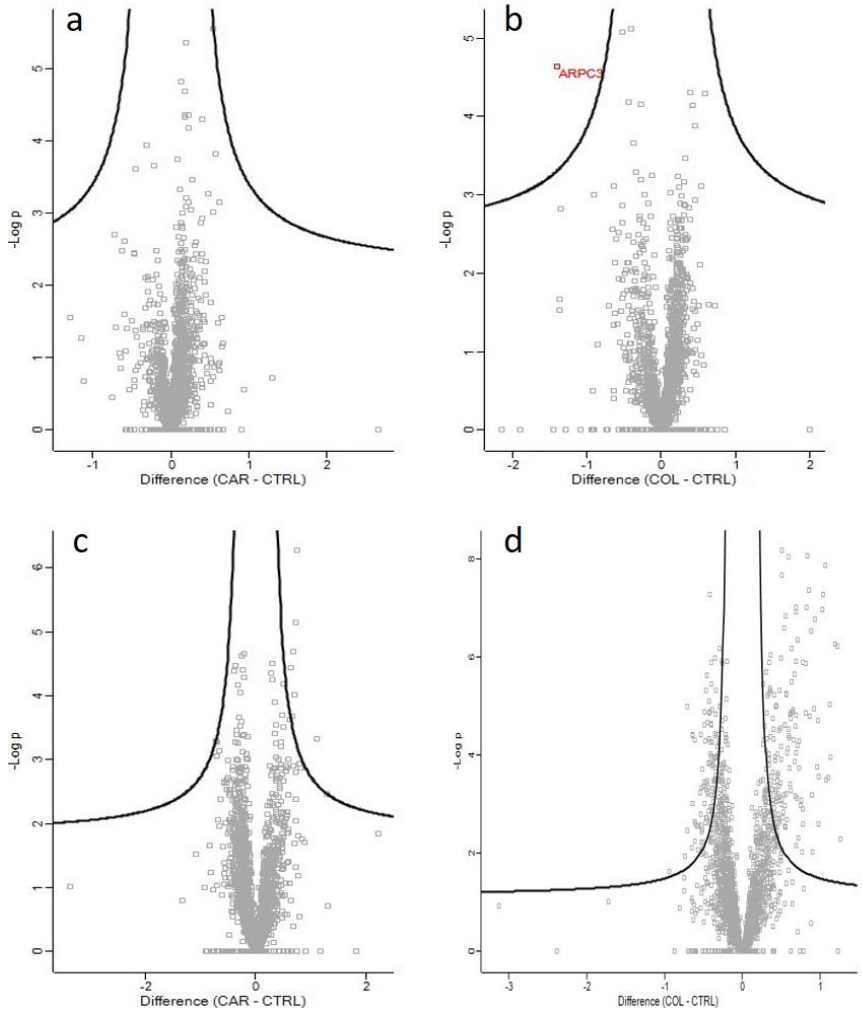

Figure 8.4*: Volcano Plot related to the proteome analysis, a) carnosine 5 µM; b) carnosinol 5 µM; c) carnosine 10 µM; d) carnosinol 10 µM*

For the treatment with 5 µM carnosine no proteins were differentially regulated. Whereas for the treatment with 5 µM carnosinol only one protein was downregulated. This protein is Actin-related protein 2/3 complex subunit 3. The Arp2/3 complex is implicated in the control of actin polymerization in cells [16].

For the treatment of carnosine and carnosinol at 10 µM, 9 and 250 differentially expressed abundant proteins were identified respectively after the Benjamini–Hochberg correction. Applying the 1.5-fold change filter, a total number of 9 and 76 proteins were finally identified (appendix).

Carnosinol treatment lead to 76 statistically different abundant proteins, 63 up and 13 downregulated. Whereas carnosine treatment lead to 9 proteins, 8 up and 1 downregulated. 8 out of 9 proteins overall identified for carnosine were also identify for carnosinol (figure 8.5-a). All 8 upregulated proteins in carnosine were also upregulated in the carnosinol treatment (figure 8.5-b), whereas the only downregulated protein was not identified in the carnosinol treatment (figure 8.5-c).

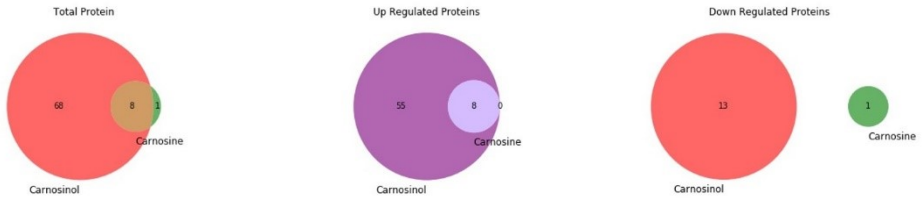

Figure 8.5*: Venn diagram of a) all proteins (Col/Car); b) up regulated (Col/Car); c) down regulated (Col/Car)*

Between the 8 co-up regulated proteins, two proteins, CKS1 and RPL22L1, are more up regulated in the carnosinol treatment than the treatment (figure 8.6).

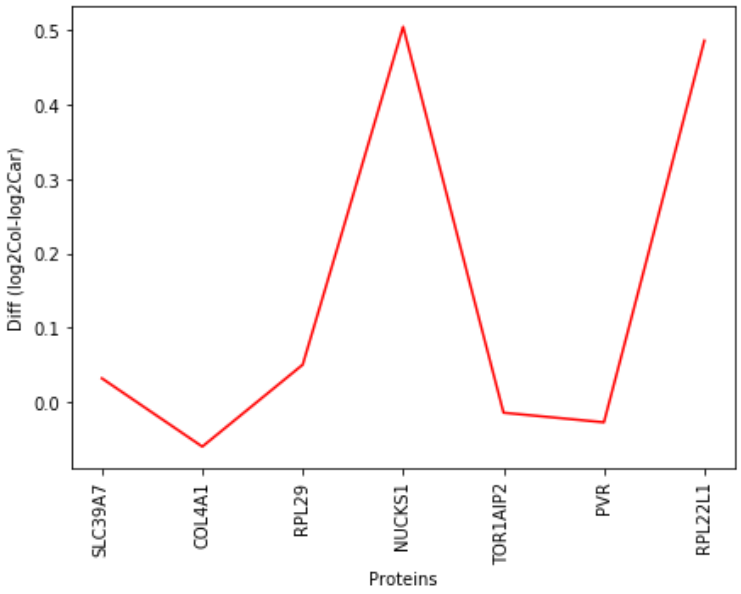

***Figure 8.6**: Differences in expression of proteins up regulated in both treatments*

To identify whether the proteins are connected to one another, a network analysis was carried out with STRING [12]. String is a web-based algorithm developed to identify protein-protein interaction networks and it's based on a database and literature, moreover it implements Gene Ontology (GO) and KEGG classification systems (i.e. classifications that aim to uniquely identify proteins and genes with a universal standard terminology) [17].

For the analysis, proteins were separated between up and down regulated and analyzed on the software. The minimum required interaction score was set as high confidence to have only reliable interactions

The Network analysis outputs are a visualization of the network identified and, if networks are identified, the relative Gene Ontology classifier with an FDR of 0.01.

Only the STRING analysis of the proteins up regulated in the carnosinol treatment reported a PPI (protein-protein interaction) enrichment p value statistically significant ($p = 0.00163$). In figure 8.7 is reported the STRING network analysis with only the associated proteins.

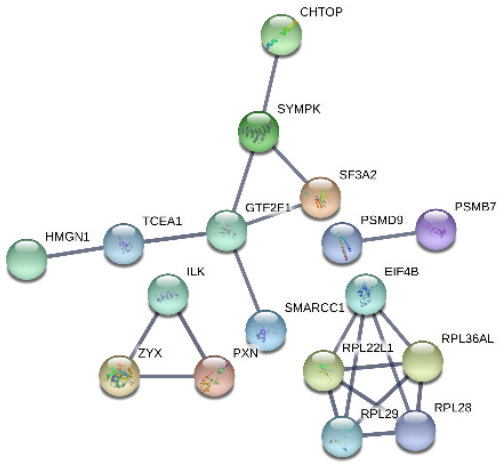

***Figure 8.7**: STRING network analysis of up regulated proteins in 10 µM carnosinol treatment*

Table 8.4 shows the Gene Ontology (cellular component) identified by the analysis corrected with an FDR of 0.05.

***Table 8.4**: Gene Ontology (cellular component) terms identified by STRING analysis for up regulated proteins with 10 μM carnosinol treatment*

#term ID	term description	observed gene count	background gene count	FDR (0.01)
GO:0005737	cytoplasm	54	11238	0.0004
GO:0044424	intracellular part	60	13996	0.0004
GO:0032432	actin filament bundle	4	57	0.0031
GO:0005634	nucleus	36	6892	0.0182
GO:0001725	stress fiber	3	50	0.0313
GO:0005829	cytosol	28	4958	0.0313
GO:0043226	organelle	52	12432	0.0313
GO:0043229	intracellular organelle	51	12193	0.0313
GO:0044464	cell part	61	16244	0.0313
GO:0071004	U2-type prespliceosome	2	17	0.0341
GO:0043231	intracellular membrane-bounded organelle	45	10365	0.04
GO:0044444	cytoplasmic part	42	9377	0.04
GO:0031981	nuclear lumen	23	4030	0.0423
GO:0032991	protein-containing complex	26	4792	0.0423
GO:0044446	intracellular organelle part	40	8882	0.0423
GO:0044428	nuclear part	24	4359	0.045
GO:1990904	ribonucleoprotein complex	8	770	0.045

Most of the terms identified are localization descriptors such as “cytoplasm”; “cytosol”; “intracellular part”; “nucleus”. Whereas few terms are giving more information such as “ribonucleoprotein complex”; “U2-type prespliceosome”, “actin filament bundle”.

Taking together the GO terms and the looking at the activity of the proteins clustered in networks it is possible to discriminate four classes (protein's gene name reported):

- Gene Transcription: EIF4B; RPL22L1; RPL36AL; RPL29; RPL28
- Protein Translation: CHTOP; SYMPK; GTF2F1; SF3A2; TCEA1; HMGN1; SMARCC1
- Cytoskeleton: ILK; ZYX; PXN
- Proteasome: PSMD9; PSMB7

Although certain networks have been identified, the analysis with more sophisticated and proprietary software like Ingenuity Pathway Analysis (Qiagen), able to identify statistically significant up or down regulation of biochemical pathways, didn't reported any statistically significant variation compared to control cell.

Looking at the protein list (appendix) one entry results to be interesting. Carnosinol treatment increases GPx4 expression with a 1.69-fold change compared to the control.

GPx4 is an important antioxidant enzyme involved in the deactivation of lipid peroxide. In chapter 4 we have evaluated the effects of carnosinol treatment in mice (WT and GPx4 +/-) fed with HFHS diet. It is safe to speculate that since carnosinol possibly increases the expression of GPx4 in healthy cells, it is likely that it happens in pathological conditions too. In this way a synergistic action will take place: activation of detoxifying enzymes and RCS sequestering. Nevertheless, tangible proof is still needed.

8.4. CONCLUSIONS

A label free quantitative proteomics analysis was carried out on HUVECs in normal conditions or treated with carnosine or carnosinol at two different concentrations (5 μM and 10 μM). The impact of the treatment on the proteome and secretome of cells was assessed. Neither carnosine nor carnosinol affect the expression of secreted proteins. At 5 μM carnosine and carnosinol do not statistically alter protein expression as detectable by proteome analysis. Only at 10 μM as a treatment concentration did we observe statistically different protein expression. Network analysis was able to identify networks only on some proteins of carnosinol treatment. Proteins involved in gene transcription, protein translation, cytoskeleton, and proteasome were clustered together, however no statistically significant activation or deactivation of any biochemical pathways was detected. Carnosine and carnosinol have little or no effect on protein expression as detectable by proteome and secretome analysis. These data are indeed consistent with several studies where, although single proteins were measured with a different technique (ELISA), carnosine treatment on control samples had no impact on protein expression [1–3]. These data indicate that carnosine and carnosinol are suitable candidates for chronic treatment since they do not have an impact (either in a positive or in a negative way) on healthy cells.

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8.6. APPENDIX

Carnosinol

-LOG(P-value)	Difference (log_2)	Majority protein IDs	Protein names	Gene names
2.298277	1.261707	B4E3J6	Arginine and glutamate-rich protein 1	FLJ10154
6.208722	1.218697	Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1
6.266317	1.195422	J3KN29	26S proteasome non-ATPase regulatory subunit 9	PSMD9
3.961574	1.135446	B4DZZ8	Zinc transporter SLC39A7	SLC39A7
5.031404	1.129175	C9JYQ9	60S ribosomal protein L22-like 1	RPL22L1
3.549566	1.104026	E5RFP0	NudC domain-containing protein 2	NUDCD2
3.486282	1.079652	A0A024R1M8	Apolipoprotein L2	APOL2
7.856625	1.069688	Q96FQ6	Protein S100-A16	S100A16
7.261137	1.033502	P06454	Prothymosin alpha	PTMA
6.970355	1.029279	C9JTE9	B-cell receptor-associated protein 29	BCAP29
3.569799	0.981741	Q9BQ61	Uncharacterized protein C19orf43	C19orf43
2.599802	0.975279	Q6UWR4	Ubiquitin-associated protein 2	UNQ610
4.693446	0.968689	J3KQ45	Trans-Golgi network integral membrane protein 2	TGOLN2
3.754049	0.959956	A6NL93	Non-histone chromosomal protein HMG-14	HMGN1
6.764249	0.929844	Q32MZ4	Leucine-rich repeat flightless-interacting protein 1	LRRFIP1
4.947654	0.918544	Q53F35	Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B
2.017588	0.901344	O60925	Prefoldin subunit 1	PFDN1
3.191464	0.895271	Q3SYF1	Sorting nexin-12	SNX12
6.515816	0.882083	Q86U62	Proteasome subunit beta type	PSMB7
4.798337	0.863932	E7EVW7	Hematopoietic lineage cell-specific protein	HCLS1

7.356652	0.853415	P15531	Nucleoside diphosphate kinase A	NME1
8.055385	0.835125	P29966	Myristoylated alanine-rich C-kinase substrate	MARCKS
7.012796	0.82837	Q9P2T1	GMP reductase 2	GMPR2
5.855717	0.811703	B4DEP6	Eukaryotic translation initiation factor 4B	EIF4B
4.849272	0.81121	Q8WW12	PEST proteolytic signal-containing nuclear protein	PCNP
5.892968	0.780202	A0A024R326	60S ribosomal protein L29	RPL29
2.902507	0.777895	Q58EY4	SWI/SNF complex subunit SMARCC1	SMARCC1
2.587156	0.775332	A8MU27	Small ubiquitin-related modifier 3	SUMO3
2.03504	0.763859	Q2Z1P3	Rab11 family-interacting protein 5	GAF1
3.075577	0.761968	B4DRG7	Condensin complex subunit 2	NCAPH
2.978348	0.745836	E7EPA1	Phosphoribosyl pyrophosphate synthase-associated protein 2	PRPSAP2
3.740882	0.733074	A0A087WTB8	Ubiquitin carboxyl-terminal hydrolase	UCHL3
4.275228	0.728191	Q5SW79	Centrosomal protein of 170 kDa	CEP170
2.304992	0.723698	Q6IBK5	General transcription factor IIF subunit 1	GTF2F1
4.199809	0.71347	K7EMT0	Splicing factor 3A subunit 2	SF3A2
6.33689	0.707938	Q969Q0	60S ribosomal protein L36a-like	RPL36AL
3.119546	0.696452	A0A087X1B7	Chromatin target of PRMT1 protein	CHTOP
4.052119	0.692052	Q59FC8	Translation initiation factor eIF-2B subunit delta	EIF2B4
7.000708	0.690293	P24390	ER lumen protein-retaining receptor 1	KDELR1
2.148992	0.690273	K7ERP4	Glutathione peroxidase	GPX4
5.910351	0.690254	A8K6V7	Ras GTPase-activating protein-binding protein 2	G3BP2
6.911135	0.687795	H0YLP6	60S ribosomal protein L28	RPL28
4.807668	0.686864	Q9NSC5	Homer protein homolog 3	HOMER3
3.158992	0.685153	Q59F15	Collagen alpha-1(IV) chain	COL4A1
4.60206	0.685089	A8K168	Malic enzyme	ME1

5.647775	0.670863	A0A024R957	Torsin-1A-interacting protein 2	TOR1AIP2
3.866406	0.669643	B4DJI2	Granulins	GRN
4.299409	0.665945	A0A087WUE9	Symplekin	SYMPK
4.910219	0.650616	A0A087WZ13	Ribonucleoprotein PTB-binding 1	RAVER1
4.873309	0.640644	A0A024R325	Succinyl-CoA ligase subunit beta	SUCLG2
6.171633	0.637096	Q15942	Zyxin	ZYX
5.482312	0.629126	V9HWC4	14 kDa phosphohistidine phosphatase	PHPT1
4.855123	0.626908	B3KSS4	Poliovirus receptor	PVR
4.749629	0.622623	V9HWF0	Integrin-linked protein kinase	ILK
3.266842	0.618271	Q9NYF8	Bcl-2-associated transcription factor 1	BCLAF1
3.397903	0.613669	P80723	Brain acid soluble protein 1	BASP1
4.822667	0.608814	F5GZ78	Paxillin	PXN
4.358174	0.599911	A6PVJ3	Endoplasmic reticulum-Golgi intermediate compartment protein 3	ERGIC3
3.739623	0.599302	A0A0S2Z3H2	Cleft lip and palate transmembrane protein 1	CLPTM1
8.026472	0.596671	Q9NZB2	Constitutive coactivator of PPAR-gamma-like protein 1	FAM120A
4.391127	0.593214	B7Z4W0	Transcription elongation factor A protein 1	TCEA1
4.522764	0.593079	Q9H7D3	Transcription factor 25	TCF25
4.128165	0.585652	A0A0A6YYJ8	Putative RNA-binding protein Luc7-like 2	LUC7L2
2.553186	-0.58531	A8YXX5	Mitochondrial carrier homolog 1	PIG60
2.597288	-0.59821	Q8TCJ8	Glutamate-rich WD repeat-containing protein 1	GRWD1
2.629434	-0.60758	Q5NKV8	Intercellular adhesion molecule 1	ICAM1
4.305193	-0.60789	O75051	Plexin-A2	PLXNA2
2.240783	-0.60795	A0A024RCB5	Chitinase domain-containing protein 1	CHID1
4.402329	-0.63548	A0A087WT20	DDB1- and CUL4-associated factor 13	DCAF13
4.418631	-0.64247	O00400	Acetyl-coenzyme A transporter 1	SLC33A1

3.020164	-0.64297	Q9H8A8	Selenium-binding protein 1	SELENBP1
2.111881	-0.64949	A0A024QYZ0	Protein transport protein Sec61 subunit gamma	SEC61G
4.98215	-0.71094	B4DKE1	All-trans-retinol 13,14-reductase	RETSAT
2.455098	-0.71152	A0A140VJG8	Catechol O-methyltransferase	COMT
1.942717	-0.73612	B4DMZ0	Non-specific protein-tyrosine kinase	CSK
1.62886	-0.94312	B7ZA00	cAMP-dependent protein kinase catalytic subunit beta	PRKACB

Carnosine

-LOG(P-value)	Difference (log_2)	Majority protein IDs	Protein names	Gene names
2.472579	1.289282	B4E3J6	Arginine and glutamate-rich protein 1	ARGLU1
3.33086	1.103495	B4DZZ8	Zinc transporter SLC39A7	SLC39A7
6.280675	0.745112	Q59F15	Collagen alpha-1(IV) chain	COL4A1
5.149687	0.729905	A0A024R326	60S ribosomal protein L29	RPL29
4.009597	0.713647	Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1
4.689782	0.685189	A0A024R957	Torsin-1A-interacting protein 2	TOR1AIP2
3.675021	0.654154	B3KSS4	Poliovirus receptor	PVR
4.435796	0.642769	C9JYQ9	60S ribosomal protein L22-like 1	RPL22L1
3.296313	-0.72864	Q6FI51	DnaJ homolog subfamily B member 1	DNAJB1

9 Conclusion

Carnosine is one of the most studied and promising sequestering agents. It reacts with RCS such as HNE, HHE, acrolein [1], malondialdehyde, methylglyoxal [2] selectively, not reacting with endogenous aldehyde such as pyridoxal [3]. L-carnosine was tested *in vitro* and *in vivo* in several models of oxidative stress diseases providing promising results in the prevention and/or regression of such pathologies [4–6]. Although its mechanism of action is still not completely understood.

In this work, hyphenated analytical methods were developed to detect carnosine and its natural and synthetic derivatives in biospecimens, as well as their adducts with reactive carbonyl species.

The implementation of a hydrophilic interaction chromatography resulted in an efficient and easy way to separates histidine dipeptides. Since this chromatography implies initial high percentage of organic solvents, it facilitates the sample preparation of biospecimens reducing the steps necessary to have a sample ready to be analyzed. The LC-MS/MS system developed resulted also versatile and it was applied in several experiments such as: tissue distribution, metabolic stability, and enzymatic activity evaluation.

A method for the chromatographic resolution of carnosine enantiomers was also developed relying on a teicoplanin-based column. The method was hyphened with both UV and MS detector. The MS detector allows a further step of separation on the molecular level, allowing to completely resolve mixture samples of carnosine and its analogues (i.e. alanine and balenine). Moreover, it allowed to identify an enantiomeric impurity of one of the standards used in the method development (D-balenine).

The application of the above-mentioned methods to biospecimen samples from carnosinol-treated mice allowed to determine the tissue distribution of the compound.

Specifically, a preferential distribution in liver and kidney was found. In liver the adduct with acrolein was identified by means of nano-LC - high resolution mass spectrometry, providing a solid proof of carnosinol RCS-sequestering activity. Moreover, for the first time it was demonstrated the metabolic instability of carnosinol-HNE adduct in tissues. Carnosine-acrolein metabolic instability was already demonstrated by Baba and colleagues, identifying Aldose Reductase as the enzyme involved in the metabolism [7]. Considering this, studies in the evaluation of the metabolic fate of carnosine adduct with other RCS should be extensively carried out to identify the real biomarker of carnosine in vivo activity toward RCS, if any.

Besides the development of carnosinase stable derivatives, a second way to exploit carnosine beneficial activity is the development of carnosinase inhibitors. Exploiting the versatility of the HILIC-MS/MS method developed, competition experiments were carried out with natural analogues of carnosine and some interesting molecules to understand the structure – activity relationship of a putative inhibitor. The experimental data were shared with computational chemistry colleagues that via means of docking/virtual screening and molecular dynamics identified 5 hit compounds to be tested as carnosinase inhibitors.

The use of carnosine to prevent catecholaldehyde toxicity has recently gained interest. The research on this topic was the focus of my visiting period at the University of Iowa under the supervision of Prof. Anderson. The reactivity of carnosine with DOPEGAL was evaluated in vitro and for the first time the carnosine-DOPEGAL Schiff base adduct was determined in biological condition. The adduct was characterized via MS/MS experiments and the reactivity of carnosine in biological environment was proved in cell lysate. Carnosine can form adduct with DOPEGAL either incubating carnosine and DOPEGAL in cell lysate or NE and carnosine in both MAO-A recombinant solution and active cell lysate. This work set the bases regarding the carnosine quenching activity of DOPEGAL and its possibly role as biomarker of cardiac diseases.

Finally, a proteomics study on the impact of carnosine and carnosinol as detectable by HUVEC cells was carried out to evaluate whether histidine dipeptides can interfere with protein expression. In the condition tested carnosine and carnosinol have little or none impact on the expression of proteins. These data do not indicate that there is no impact at all, but that the modifications, if any, are not detectable on healthy cells. This property is interesting especially for treatment that are supposed to be chronic, making carnosine and carnosinol ideal candidates. In the future the impact of carnosine and carnosinol on pathological model should be carried out to investigate their activity in such environment.

Even if the mechanism of activity of histidine dipeptides is still not completely clear this work makes advancement in its understanding. The quenching activity on RCS was identify in vivo, even if more knowledge on the metabolic fate of the adducts is needed. A new method for the direct determination of carnosinase activity was developed and a new quenching target has been identified (i.e. DOPEGAL), setting the base of a new sub-field of research for histidine dipeptides.

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10 Scientific Curriculum Vitae

I was the representant of the XXXIII cycle of PhD students for the PhD program in Pharmaceutical Sciences. From December 2018 to March 2020 I was the representant for Pharmaceutical Sciences in the PhD Council and from January 2019 to March 2020 I was also the Vice President of the PhD Council.

TUTORING

During my program I supervised the laboratory activities of eight master students (pharmacy, CTF and pharmaceutical biotechnology), a visiting master student from France, visiting PhD Students (Austria, Belgium, Nederland, Italy) and I collaborated with a visiting senior post-doc from Brazil.

I was the co-supervisor of six experimental master thesis in pharmaceutical chemistry (CTF), pharmacy and pharmaceutical biotechnology.

RESEARCH PROJECTS

I am part of the Research Unit headed by Prof Luca Regazzoni, awarded by the PRIN 2017 for the research project entitled: "*Linking tryptophan catabolism to amyotrophic lateral sclerosis: from pathogenesis to the pharmacological treatment*" protocol n° 20173EAZ2Z; P.I. Claudia Volpi Ph.D, Università degli Studi di Perugia.

Presenting a Research Project, I was awarded of 100000 hours form the UNITECH Platform INDACO of the Università degli Studi di Milano and I used these hours in collaboration with the XXXIII cycle PhD Student Silvia Gervasoni to work on part of my thesis. Results obtained from the use of the platform were included in a paper (doi: https://doi.org/10.1016/j.jpba.2020.113440)

SCHOLARSHIPS

From March 2020 to August 2020 I was a visiting scholar at The University of Iowa, at the department of Pharmaceutical Sciences and Experimental Therapeutics, under the supervision of Prof. Anderson.

DISSEMINATION ACTIVITIES

I have participated at the following summer school:

- Summer School in Pharmaceutical Analysis (SSPA) from the 19th to the 21st of September 2018 – Rimini, Italy
- Summer School in Pharmaceutical Analysis (SSPA) from the 12th to the 13th of September 2019 – Pescara, Italy

I participated at several national and international congresses presenting either a poster or a communication:

- Analytical methods to study oxidative damage, antioxidants and drugs, 24-26 May 2018 Bialystok, Poland
- 32nd International Symposium on Chromatography 23-27 September 2018 Cannes-Mandelieu France
- MS-PharmaDay, 24-26 October 2018, Colleretto Giacosa, Italy.
- 7th Ms J Day: I giovani e la spettrometria di massa, 27 May 2019, Bolzano, Italy.
- NNMC XXVI, 16-19 July 2019 Milan, Italy
- Recent development in pharmaceutical analysis (RDPA) 8-11 September 2019, Pescara, Italy
- MYCS, 25-27 November 2019, Rimini, Italy
- ASMS Reboot, 1-12 June 2020, online
- 5th Chicago Mass Day, 14 August 2020, online
- Massa VIP 2020, September 2020, online

I performed tutoring activity in the laboratory of Master Courses:

- Laboratorio analisi dei farmaci I (CTF)
- Laboratorio analisi quantitativa (Farmacia)
- Laboratorio analisi dei farmaci II (CTF)
- Laboratorio analisi qualitativa (Farmacia)
- Laboratorio analisi Tossicologica (STE)
- Laboratorio di analisi fito-farmaceutiche (STE)
- Analisi avanzata dei principi attivi delle droghe vegetali (STE)

Posters

- **Ettore Gilardoni**, Luca Regazzoni, Giancarlo Aldini "*An analytical method to measure histidine dipeptide adducts in biological matrices*" **Analytical methods to study oxidative damage, antioxidants and drugs** 24-26 May 2018 Bialystok, Poland
- Igor Petkovic, **Ettore Gilardoni**, Bresgen Nikolaus, Luca Regazzoni, Giancarlo Aldini, Peter Eckl "*Determination of AGE-dependent intracellular HNE metabolism by using LC/ESI-MS*" **Analytical methods to study oxidative damage, antioxidants and drugs** 24-26 May 2018 Bialystok, Poland
- Laura Fumagalli, **Ettore Gilardoni** "*Sintesi "green" di un nuovo antimicrobico"* **6° workshop nazionale gruppo interdivisionale di Green Chemistry-Chimica Sostenibile** 15 June 2018 Milan, Italy
- Laura Fumagalli, **Ettore Gilardoni**, Claudia Picozzi, Giancarlo Aldini, Giulio Vistoli, Marina Carini "*Para-halo-substituted analogues of domiphen bromide as novel antimicrobial agent*" **Italian-Spanish-Portuguese Joint Meeting in Medicinal Chemistry MedChemSicily2018,** 17-10 July 2018 Palermo, Italy

- Helena Macut, Xiao HU, Delia Tarantino, Luca Regazzoni, **Ettore Gilardoni**, Sara Pellegrino, Maria Luisa Gelmi "*Design, synthesis and in vitro evaluation of PFKFB3 phosphatase activity allosteric modulator*" **XXV EFMC International Symposium on Medicinal Chemistry** 2-6 September 2018, Ljubljana, Slovenia

- **Ettore Gilardoni**, Laura Fumagalli, Lucia Pucciarini, Veronica Marrone, Roccaldo Sardella, Marina Carini, Giancarlo Aldini, Luca Regazzoni, "*Enantioselective Chromatography for the Determination of Histidine Dipeptides in Food and Food Supplements*" **32nd International Symposium on Chromatography** 23-27 September 2018 Cannes-Mandelieu France.

- Maspero Marco, **Gilardoni Ettore**, Regazzoni Luca; Aldini Giancarlo. Carini Marina, De Amici Marco, Dallanoce Clelia "*Synthesis and application of isotope labelled carnosine in LCMS/MS*" **National Meeting in Medicinal Chmistry XXVI** 16-19 July 2019 Milan, Italy

- **Ettore Gilardoni**, Helena Macut, Delia Tarantino, Luca Regazzoni, Sara Pellegrino, Maria Luisa Gelmi and Marina Carini "*Measurement of PFKFB3 phosphatase activity by means of liquid chromatography-tandem mass spectrometry*" **Recent Development in Pharmaceutical Analysis** 8-11 September 2019 Pescara Italy.

Oral Communications:

- Laura Fumagalli, **Ettore Gilardoni** "*Sintesi "green" di un nuovo antimicrobico"* **6° workshop nazionale gruppo interdivisionale di Green Chemistry-Chimica Sostenibile** 15 June 2018 Milan, Italy

- **Ettore Gilardoni**, Laura Fumagalli, Lucia Pucciarini, Veronica Marrone, Roccaldo Sardella, Marina Carini, Giancarlo Aldini, Luca Regazzoni, "*Enantioselective Chromatography for the Determination of Histidine Dipeptides in Food and Food Supplements*" **32nd International Symposium on Chromatography** 23-27 September 2018 Cannes-Mandelieu France.

- **Ettore Gilardoni**, Giulio Vistoli, Marina Carini, Giancarlo Aldini, Luca Regazzoni, "*A Stable carnosine derivative confirms the mechanism of action of histidine dipeptides as carbonyl stress mitigators"* **10th MS Pharmaday** 24-26 October 2018 Colleretto Giacosa, Italy

- **Ettore Gilardoni,** Marco Maspero, Clelia Dallanoce, Giancarlo Aldini, Marina Carini, Luca Regazzoni "*Set up of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the measurement of serum carnosinase activity*" **Recent Development in Pharmaceutical Analysis** 8-11 September 2019 Pescara Italy.

- **Ettore Gilardoni**, Giancarlo Aldini, Alfonsina D'Amato, and Luca Regazzoni "*Development and evaluation of the mechanism of action of a carnosine derivative in oxidative stress-based diseases*" **Merck Young Chemists' Symposium** 25-27 November 2019 Rimini Italy

- **Ettore Gilardoni**, Matteo Mori, Stefania Villa, Arianna Gelain, Luca Regazzoni "*Identification of the mechanism of action of 5,6-dimethyl-1H,3H-2,1,3-benzothiadiazole-2,2-dioxide as STAT3 inhibitor*" **5th Chicago Mass Spec Day**, 14 August 2020, virtual meeting.

- **Ettore Gilardoni**, Angelica Artasensi, Laura Fumagalli, Alfonsina D'Amato, Marina Carini, Giancarlo Aldini, Luca Regazzoni," *A direct measurement of serum carnosinase activity for the discovery of competitive inhibitors"*, **Massa VIP 2020**, September 2020, virtual symposium.

Papers:

- Laura Fumagalli, Lucia Pucciarini, Luca Regazzoni, **Ettore Gilardoni**, Marina Carini, Giulio Vistoli, Giancarlo Aldini and Roccaldo Sardella: "*Direct HPLC separation of carnosine enantiomers with two chiral stationary phases based on penicillamine and teicoplanin derivatives*" **Journal of Separation Science**. doi: 10.1002/jssc.201701308.

- Laura Fumagalli, Alessandra Moretto, **Ettore Gilardoni**, Claudia Picozzi, Giulio Vistoli, Marina Carini; *Data on thermal and hydrolytic stability of both domiphen bromide and para-bromodomiphen bromide*". **Data Brief** doi: 10.1016/j.dib.2018.08.152

- Ethan J. Anderson, Giulio Vistoli, Lalage A. Katunga, Katsuhiko Funai, Luca Regazzoni, T. Blake Monroe, **Ettore Gilardoni**, Luca Cannizzaro, Mara Colzani, Danilo De Maddis, Giuseppe Rossoni, Renato Canevotti, Stefania Gagliardi, Marina Carini, and Giancarlo Aldini:"*A Carnosine Analog Mitigates Metabolic Disorders of Obesity by Reducing Carbonyl Stress*". **Journal of Clinical Investigation**. doi: 10.1172/JCI94307

- Inge Everaert, Giovanna Baron, Silvia Barbaresi, **Ettore Giardoni**, Crescenzo Coppa, Marina Carin, Giulio Vistol, Tine Bex,Jan Stautemas, Laura Blancquaert, Wim Derave, Giancarlo Aldini, Luca Regazzoni: "*Development and validation of a sensitive LC-MS/MS assay for the quantification of anserine in human plasma and urine and its application to pharmacokinetic study*." **Amino Acid** doi: 10.1007/s00726-018-2663-y

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- Helena Macut, Xiao Hu, Delia Trantino**, Ettore Gilardoni**, Francesca Clerici, Luca Regazzoni, Alessandro Contini, Sara Pellegrino, Maria Luisa Gelmi "*Tuning PFKFB3 Bisphosphatase Activity Through Allosteric Interference*" **Scientifc Reports** doi: 10.1038/s41598-019-56708-0.

- Igor Petkovic, Nikolaus Bresgen, **Ettore Gilardoni**, Luca Regazzoni, Koji Uchida, Giancarlo Aldini, Werner Siems, Peter Eckl "*In Vitro Aging of Human Skin Fibroblasts: Age-Dependent Changes in 4-Hydroxynonenal Metabolism*" **Antioxidants** doi: 10.3390/antiox9020150.

- **Ettore Gilardoni**, Silvia Gervasoni, Marco Maspero, Clelia Dallanoce, Giulio Vistoli, Marina Carini, Giancarlo Aldini, Luca Regazzoni, "*Development of a direct LC-ESI-MS method for the measurement of human serum carnosinase activity*" **Journal of Pharmaceutical and Biomedical Analysis** doi: https://doi.org/10.1016/j.jpba.2020.113440

- Matteo Mori, **Ettore Gilardoni**, Luca Regazzoni, Alessandro Pedretti, Diego Colombo, Gary Parkinson, Akira Asai, Fiorella Meneghetti, Stefania Villa, Arianna Gelain, "Towards the Inhibition of Protein–Protein Interactions (PPIs) in STAT3: Insights into a New Class of Benzothiadiazole Derivatives" **Molecules**, doi: 10.3390/molecules25153509

§ equally contributed

Award

- AFSEP Award for the best mini oral presentation at the **32nd International Symposium on Chromatography** 23-27 September 2018 Cannes-Mandelieu France.