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Author/s:

Mayneris-Perxachs, J;Meikle, P;Mousa, A;Naderpoor, N;Fernández-Real, JM;de Courten, B

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Novel Relationship Between Plasmalogen Lipid Signatures and Carnosine in Humans

Jordi Mayneris-Perxachs, Peter Meikle, Aya Mousa, Negar Naderpoor, José Manuel Fernández-Real, and Barbora de Courten*

Introduction: Carnosine is a naturally occurring dipeptide abundant in the skeletal and cardiac muscle and brain, which has been shown to improve glucose metabolism and cardiovascular risk. This study showed that carnosine supplementation had positive changes on plasma lipidome. Here, this study aimed to establish the relationship of muscle carnosine and serum carnosinase-1 with cardiometabolic risk factors and the lipidome.

Methods and Results: This study profiles >450 lipid species in 65 overweight/obese nondiabetic individuals. Intensive metabolic testing is conducted using direct gold-standard measures of adiposity, insulin sensitivity and secretion, as well as measurement of serum inflammatory cytokines and adipokines. Muscle carnosine is negatively associated with 2-h glucose concentrations, whereas serum carnosinase-1 levels are negatively associated with insulin sensitivity and positively with IL-18. O-PLS and machine learning analyses reveal a strong association of muscle carnosine with ether lipids, particularly arachidonic acid-containing plasmalogens. Carnosinase-1 levels are positively associated with total phosphatidylethanolamines, but negatively with lysoalkylphosphatidylcholines, trihexosylceramides, and gangliosides. In particular, alkylphosphatidylethanolamine species containing arachidonic acid are positively associated with carnosinase-1.

Conclusion: These associations reinforce the role of muscle carnosine and serum carnosinase-1 in the interplay among low-grade chronic inflammation, glucose homeostasis, and insulin sensitivity.

1. Introduction

Type 2 diabetes (DM2) is a major public health challenge because of its high and increasing prevalence and significant adverse effects on health.^[1] DM2 contributes to high morbidity and mortality through diabetes complications, primarily cardiovascular disease (CVD), which is the leading cause of death worldwide, including in those with DM2.^[2] Together, DM2 and CVD impose a heavy financial and patient burden across the entire healthcare system.^[1–3]

Carnosine (β -alanyl-L-histidine), a naturally occurring dipeptide and over-the-counter food supplement, has recently emerged as a potential strategy for reducing DM2 and CVD risk factors.^[4,5] Carnosine is mainly found in human skeletal muscle and carnosine supplementation is shown to improve muscle performance and exercise endurance.^[6] Lower concentrations of carnosine are also found in other tissues and cells such as the brain, stomach, and erythrocytes.^[7] Male gender, younger age, and meat consumption are associated with higher muscular concentrations of carnosine in humans.^[8] In

J. Mayneris-Perxachs, J. M. Fernández-Real
 Department of Endocrinology
 Diabetes and Nutrition
 Hospital of Girona “Dr Josep Trueta,” University of Girona
 Girona Biomedical Research Institute (IdibGi)
 Girona, Spain


J. Mayneris-Perxachs, J. M. Fernández-Real
 CIBERobn Pathophysiology of Obesity and Nutrition
 Instituto de Salud Carlos III
 Madrid, Spain

P. Meikle
 Baker Heart and Diabetes Institute
 Melbourne, Australia

A. Mousa, N. Naderpoor
 Monash Centre for Health Research and Implementation
 Monash University
 Melbourne, Australia

J. M. Fernández-Real
 Department of Medical Sciences
 Faculty of Medicine
 University of Girona
 Girona, Spain

B. de Courten
 Department of Medicine
 School of Clinical Sciences
 Monash University
 Melbourne, Australia
 E-mail: jmfreal@idibgi.org; barbora.decourten@monash.edu

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/mnfr.202100164>

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addition to previously known benefits of carnosine for muscle function, more recent animal studies have shown that carnosine reduces obesity and improves glucose metabolism, chronic low-grade inflammation, lipid levels and peroxidation, atherosclerotic plaque instability, blood pressure, insulin resistance, and insulin secretion, along with increasing β -cell mass.^[4,9–15] We and others have also shown beneficial effects of carnosine supplementation on glucose and lipid metabolism in humans.^[5,16,17] Molecular and animal research suggests that the beneficial effects of carnosine on these risk factors occur, at least in part, through its anti-inflammatory, anti-oxidative, and anti-glycating properties.^[5,11,12,16,17]

Dietary carnosine is usually rapidly degraded to β -alanine and histidine upon absorption. However, carnosine catabolism is less pronounced in individuals with low serum carnosinase-1 (CN-1) activity, resulting in increases in circulating carnosine.^[18] CN-1 is the only dipeptidase with substrate specificity for carnosine, anserine and homocarnosine.^[19] Therefore, potential benefits from carnosine supplementation may be counteracted by high serum CN-1 activity. Due to their abundance in human skeletal muscle and serum, respectively, skeletal muscle carnosine and serum CN1 are two key players involved in carnosine metabolism.

One of the key metabolic disturbances associated with the development of DM2 and CVD is dyslipidemia. While conventional lipid profiling is helpful for the diagnosis and management of dyslipidemia, recently developed lipidomics approaches can provide new insights into lipid metabolism by offering more detailed measures of lipid classes and species in plasma. Lipidomics enables the comprehensive identification and quantification of all lipids from serum, plasma, tissue, whole organism, or cell. Lipidomics techniques have shown promising results in the identification of metabolic biomarkers, understanding the mechanisms of lipid metabolism underlying disease pathogenesis, as well as in determining responses to therapeutic interventions. Recent studies have demonstrated associations between plasma lipid classes and species and DM2 and CVD.^[20,21] In particular, plasmalogens are a special class of lipids with unique properties. They contain a vinyl-ether alkyl chain in the *sn*-1 position and a long-chain *n*-3 or *n*-6 PUFA at the *sn*-2 position. Due to this unique structure, they modulate membrane fluidity, possess antioxidant properties, and act as reservoirs of inflammatory active lipid mediators.^[22] Indeed, plasmalogen deficiency has been associated with cardiometabolic disorders such as obesity^[23] and DM2^[20] in humans.

Our previous pilot clinical trial investigated the effects of carnosine supplementation on DM2 risk factors, including the human plasma lipidome and showed beneficial effects on trihexosylceramide and phosphatidylcholine as well as an association between serum CN-1 activity and trihexosylceramide.^[24] Given the compelling evidence from animal studies, and the paucity of human data on the benefits of carnosine in glucose and lipid metabolism, further exploration of carnosine in relation to cardiometabolic risk factors and lipidomic profiles is warranted. Hence, this study aims to explore whether human muscle carnosine content and CN-1 (an enzyme hydrolyzing carnosine into its amino acids) may be associated with metabolic and cardiovascular risk factors including the plasma lipidome in a well-characterized cohort of overweight or obese individuals.

2. Results

Baseline characteristics of the participants are described in **Table 1**. The study population included 65 overweight or obese participants aged 18–57 years old. Obese participants had lower carnosine and insulin sensitivity and higher serum leptin concentrations compared to overweight individuals. After controlling for age, sex, and fat mass, muscle carnosine levels were significantly associated with 2-h glucose concentrations after the 75g OGTT ($r = 0.27$, $p = 0.05$). In particular, participants in the higher quartile of carnosine levels had the highest 2-h glucose concentrations (**Figure 1A**). The serum concentration of CN-1 was positively associated with IL-18 ($r = 0.30$, $p = 0.02$), but not with other inflammatory cytokines (**Figure S1**, Supporting Information). Conversely, it was negatively associated with adiponectin levels ($r = -0.32$, $p = 0.013$) and insulin sensitivity ($r = -0.31$, $p = 0.017$) (**Figure 1B** and **Figure S1**, Supporting Information).

O-PLS regression models were built using the lipid classes and species to identify lipids associated with muscle carnosine levels. Significant O-PLS models were obtained after permutation testing (**B**). Based on O-PLS correlation-scaled loadings, seven lipid classes were selected (**Figure 2C**). Notably, all the lipid classes and subclasses associated with muscle carnosine levels were ether lipids. They constitute a subset of glycerophospholipids where the ester-carbonyl group in the *sn*-1 position has been substituted by an ether-alkyl [indicated by (O)] or vinyl ether-alkenyl [indicated by (P)] bond, with the latter known as plasmalogens.^[25] Specifically, PC, PE, LPC, and LPE plasmalogens had the strongest positive associations with muscle carnosine, followed by their alkyl ethers. These results were further validated using machine learning. The random forest-based *vita* algorithm also identified the above plasmalogens as the most important features associated with muscle carnosine levels. However, we were not able to identify their alkyl counterparts using this technique, although total TG was identified as being positively associated with carnosine. A significant O-PLS model was also obtained when using the lipid species as predictor variables (**Figure 2B**), which identified 33 lipid species. In line with the previous results, the lipid species most associated with muscle carnosine levels included several plasmalogen species [mainly PE(P), PC(P), but also LPE(P)] and some alkyl-ether species [mainly PE(O)] (**Figure 2E**). The *sn*-2 position of all these lipid species was enriched mainly with long-chain *n*-6 fatty acids, mainly arachidonic acid (C20:4 *n*-6, AA) and docosapentaenoic acid (C22:5 *n*-6, DPA). Other lipid species positively associated with muscle carnosine levels included PI, PC, PE, LPC, LPI, and DG that contained mainly AA chains. Relevant lipid species were also identified using machine learning techniques. As observed from the analysis (**Figure 2F**), the lipid species most associated with muscle carnosine included mainly PC(P) plasmalogens with AA in the *sn*-2 position. Consistent with the O-PLS results, several AA-containing PI, PC, LPC, LPI, and DG were positively associated with muscle carnosine. Using this method, we were also able to identify several ceramide and TG species linked to increased muscle carnosine levels.

No significant O-PLS models were able to predict serum CN-1 levels from either the lipids classes or lipid species. However,

Table 1. Baseline characteristics of the participants.

	Overweight (N = 30)	Obese (N = 35)	Total (N = 65)	p
Carnosine [mmol kg ⁻¹ WW]	5.64 (5.05, 7.33)	4.39 (3.89, 5.28)	4.81 (4.08, 6.02)	0.002
CN-1 activity [μmol mL ⁻¹ h ⁻¹]	1.53 (1.25, 1.81)	1.55 (1.24, 1.77)	1.54 (1.24, 1.79)	0.967
Age [years]	31.5 (24.2, 36.7)	29.0 (25.0, 35.5)	30.0 (25.0, 36.0)	0.911
Female	5 (16.7%)	19 (54.3%)	24 (36.9%)	0.002
BMI [kg m ⁻²]	27.72 (26.72, 28.95)	33.18 (30.72, 36.98)	30.15 (27.79, 33.31)	< 0.001
Body fat (%)	35.00 (29.73, 38.80)	47.70 (37.65, 50.50)	38.50 (33.80, 48.00)	< 0.001
WHR	0.95 (0.91, 0.98)	0.93 (0.90, 0.99)	0.94 (0.90, 0.99)	0.869
SBP [mmHg]	119.53 (12.00)	122.34 (12.87)	121.05 (12.46)	0.369
DBP [mmHg]	77.70 (8.71)	82.51 (7.97)	80.29 (8.60)	0.023
<i>Glucose metabolism</i>				
Fasting glucose [mmol L ⁻¹]	4.55 (4.29, 4.97)	4.48 (4.29, 4.73)	4.52 (4.29, 4.85)	0.558
2-h Glucose [mmol L ⁻¹]	5.25 (4.54, 6.29)	5.39 (4.44, 6.07)	5.30 (4.48, 6.19)	0.911
M-value [mg kg ⁻¹ min ⁻¹]	7.79 (2.87)	5.48 (2.32)	6.55 (2.82)	< 0.001
Fasting Insulin [mIU L ⁻¹]	8.10 (6.71, 9.70)	10.45 (6.67, 16.15)	8.85 (6.65, 12.80)	0.061
<i>Lipid metabolism</i>				
TC [mmol L ⁻¹]	4.85 (0.76)	4.98 (0.95)	4.92 (0.87)	0.608
TG [mmol L ⁻¹]	1.35 (1.08, 2.28)	1.30 (1.00, 1.80)	1.30 (1.00, 1.90)	0.633
HDL [mmol L ⁻¹]	1.10 (1.00, 1.35)	1.10 (1.10, 1.30)	1.10 (1.00, 1.33)	0.496
LDL [mmol L ⁻¹]	2.98 (0.61)	3.11 (0.77)	3.06 (0.71)	0.525
LDL/HDL	2.67 (0.72)	2.70 (0.85)	2.69 (0.80)	0.900
<i>Diet</i>				
Energy [kJ]	7800.4 (6950.9, 9190.8)	8356.3 (6759.0, 9925.3)	8243.2 (6763.9, 9668.3)	0.390
Protein [g]	86.80 (61.09, 105.19)	89.37 (74.81, 104.93)	88.98 (70.03, 105.02)	0.590
Total fat [g]	72.06 (54.38, 80.74)	82.65 (60.75, 91.70)	74.31 (56.13, 89.34)	0.066
Saturated fat [g]	23.21 (18.30, 31.32)	29.57 (22.00, 40.18)	26.57 (20.62, 33.45)	0.045
Carbohydrate [g]	222.99 (59.59)	233.99 (84.99)	228.79 (73.62)	0.585
<i>Adipokines</i>				
Adiponectin [ng mL ⁻¹]	3366.6 (1532.4, 11453.2)	5135.9 (3258.2, 11013.8)	4749.3 (2348.0, 11243.4)	0.236
<i>Inflammatory markers</i>				
hsCRP [mg L ⁻¹]	2.50 (1.00, 5.80)	1.70 (0.90, 4.30)	1.95 (0.90, 4.45)	0.594
TNFα [pg mL ⁻¹]	32.02 (14.20, 75.88)	32.36 (21.80, 62.69)	32.36 (16.93, 63.47)	0.787
MCP-1 [pg mL ⁻¹]	542.1 (385.2, 1129.8)	761.1 (481.9, 967.3)	631.6 (425.43, 98.8)	0.286
IL-6 [pg mL ⁻¹]	22.59 (11.83, 44.04)	22.13 (14.09, 42.43)	22.13 (13.05, 44.38)	0.911
IL-10 [pg mL ⁻¹]	8.43 (6.30, 16.91)	9.66 (6.71, 14.85)	8.72 (6.45, 16.34)	0.767
IL-18 [pg mL ⁻¹]	175.25 (103.71, 259.40)	174.60 (121.19, 264.92)	174.74 (108.7, 264.7)	0.844

Values are expressed as means ± SD for normally distributed variables, median (IQR) for non-normally distributed variables, and counts (%) for categorical variables. *p* were obtained using a one-way ANOVA, a Kruskal-Wallis test, or χ^2 test for normally-, non-normally distributed, and categorical variables, respectively. BMI, body mass index; CN-1, carnosinase-1; WHR, waist to hip ratio; hsCRP, high-sensitivity C-reactive protein; TC, total cholesterol; TG, triglycerides; HDL/LDL, high/low-density lipoprotein cholesterol; SBP/DBP, systolic/diastolic blood pressure; MCP-1, monocyte chemoattractant protein-1; TNF, tumour necrosis factor; M-value, insulin sensitivity by hyperinsulinaemic-euglycaemic clamp; TC, total cholesterol; IL, interleukin.

due to the high-dimensionality of the data, usually a large proportion of the features are uninformative in relation to the biological question under study, leading to decreased model performance efficiency. Therefore, we applied a random-forest based machine learning variable selection approach. The *vita* algorithm selected four lipid species associated with serum CN-1 concentrations (**Figure 3A**). Specifically, total PE were significantly positively associated with CN-1, whereas total LPC(O), Hex3Cer, and GM were negatively associated. In line with these results, machine learning analyses selected several PE species positively associated with CN-1 levels. In addition, some PE(O)

alkyl ethers containing AA in the *sn*-2 position were also positively associated with serum CN-1 concentrations. Selected lipid species which were negatively associated with CN-1 included LPC(O) alkyl-ethers containing long-chain saturated fatty acids (C20:0-C24:0), several LPC containing C18:1, C18:2, and C20:2 fatty acids, and GM3 and Hex3Cer with C18:0, C22:0, and C24:1 fatty acids. Notably, LPC(P) and LPC(O) containing eicosanoic acid (arachidic acid) were among the most important features associated negatively with CN-1.

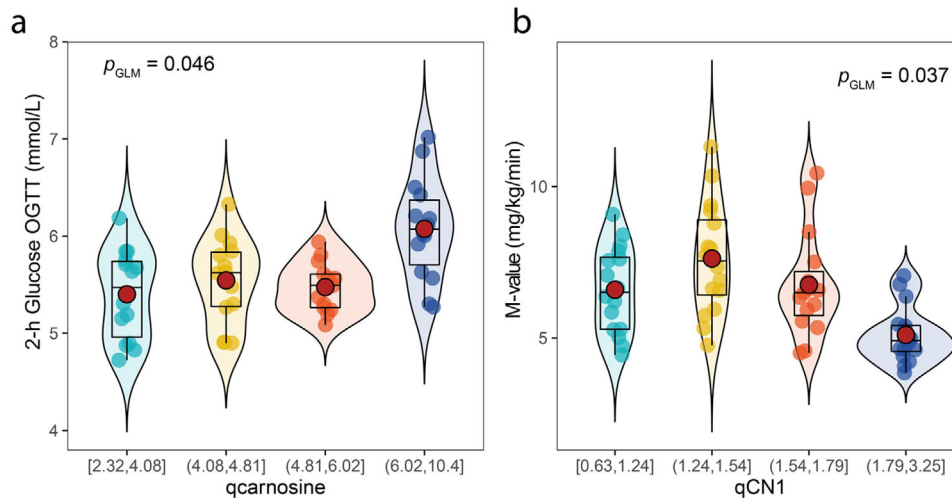


Figure 1. Associations of muscle carnosine and plasma CN-1 with glucose and insulin metabolism. **A)** Violin plots for the associations of 2-h glucose from the oral glucose tolerance test according to the muscle carnosine quartiles. **B)** Insulin sensitivity measured with the by hyperinsulinaemic-euglycaemic clamp according to the plasma CN-1 levels

3. Discussion

Carnosine has recently emerged as a promising new approach to tackle DM2 and CVD risk factors.^[4,5] In a previous pilot study by our group involving 24 overweight and obese adults, we found that carnosine supplementation maintained total Hex3Cer, PC, and free cholesterol compared to placebo.^[24] Here, we found strong associations among plasmalogens, specifically those containing mainly AA chains, and muscle carnosine levels. To our knowledge, this is the first study to examine muscle carnosine in relation to lipidomics and report relationships between muscle carnosine and plasmalogens. We also found that serum CN-1 was negatively associated with LPC(O) and total Hex3Cer. This supports the results of our pilot study, where we also found that Hex3Cer was inversely related with CN-1 activity.^[24]

One of the most remarkable findings of this study was that all the lipid classes and subclasses associated with muscle carnosine levels were ether lipids. Unlike conventional glycerophospholipids that have fatty acyl chains in the *sn-1* and *sn-2* positions of the glycerol backbone by an ester linkage, ether lipids are characterized by an ether bond, usually in the *sn-1* position, and an ester bond in the *sn-2* position. PC, PE, LPC, and LPE plasmalogens had the strongest positive associations followed by their alkyl ethers. Plasmalogens are critical for human health and have established roles in neuronal development, immune responses and as endogenous antioxidants, while they also take part in the development of insulin resistance and atherosclerosis.^[26,27]

The long chain fatty acid alcohols in the *sn-1* position of ether lipids are mainly restricted to C16:0, C18:0, or C18:1. In the particular case of plasmalogens, the *sn-2* positions are enriched with polyunsaturated fatty acids, mainly AA (C20:4, AA *n-6*) or docosahexaenoic acid (C22:6 *n-3*, DHA).^[26,28] Of note, the lipid species that had the strongest associations with serum CN-1 were PE(O-38.5) and PE (16:0/22:6), while with muscle carnosine were PC(P-16:0/20:4) and PC(P-15:0/20:4), both containing AA in the *sn-2* position, a precursor for eicosanoid biosynthesis (prostaglandins, thromboxane, leukotrienes), which are im-

portant inflammatory mediator molecules.^[29,30] Interestingly, we also found positive associations between CN-1 and serum IL-18 levels, but not with other cytokines. This is consistent with previous that found positive correlations between IL-18 serum levels and serum ferritin, but not with CRP, IL-6, or IL-1 β levels.^[31] In fact, the IL-1B/IL-6/CRP and IL-18/ferritin inflammatory programs have been recently proposed as two different inflammatory responses mechanism.^[32] In a study of twin pairs discordant for obesity, obese twins had increased levels of adipose tissue PE(P) containing AA, making adipocytes more prone to inflammatory responses.^[33] As both the vinyl ether bond in plasmalogens and the long-chain PUFA are particularly susceptible to oxidation, plasmalogens are also suggested to play an important role as antioxidants, protecting other lipids from oxidative stress.^[26,28] Therefore, the strong association between PC(P-16:0/20:4) and muscle carnosine could be related to an off-set mechanism due to the antioxidant properties of carnosine and reflect an antioxidant response to increased oxidative stress in individuals with insulin resistance. In fact, muscle histidine-containing dipeptides (such as carnosine) are known to be elevated by glucose intolerance in both rodents and men, implying that increased muscle carnosine could constitute a compensatory mechanism aimed at preventing cell damage in states of impaired glucose tolerance.^[34]

The main limitation of the present study is its cross-sectional design, which means that we cannot establish causality or rule out reverse causality. Moreover, the sample size may have been too small to detect relationships with some lipid species or classes and our results may not be generalized to other populations as we only included overweight/obese individuals. The findings should be confirmed in longitudinal studies with larger cohorts of participants including lean individuals. Despite these limitations, this is the first study to examine associations between muscle carnosine and CN-1 with human lipidomic profiles. We report a comprehensive assessment of the human lipidome (>450 species) in a well-characterized cohort with gold-standard measures of adiposity and insulin sensitivity and with no confounding factors

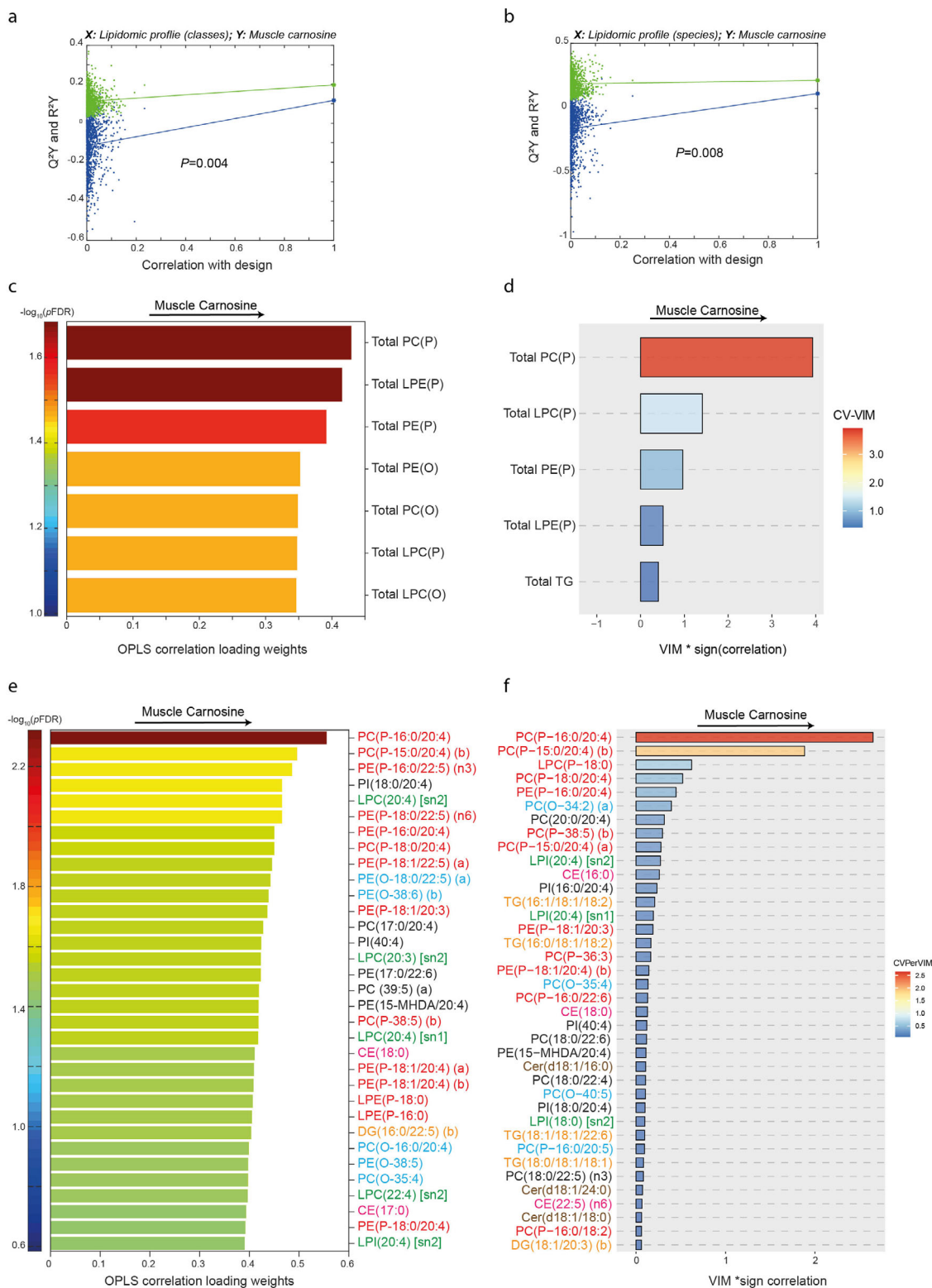


Figure 2. Associations of lipidomics profiles with muscle carnosine. **A)** Goodness of fit (R^2) and goodness of prediction (Q^2) parameters obtained from a leave-one-out cross-validated O-PLS regression model predicting muscle carnosine from the lipidomic classes and **(B)** species profiles. **C)** O-PLS correlation loading weights for the lipid classes associated with the muscle carnosine levels. **D)** Cross-validated permutation variable importance (CVPVI) measure \times sign of the correlation between each lipid class associated with muscle carnosine levels. **E)** O-PLS correlation loading weights for the lipid species associated with the muscle carnosine levels. **F)** Cross-validated permutation variable importance (CVPVI) measure \times sign of the correlation between each lipid species associated with muscle carnosine levels

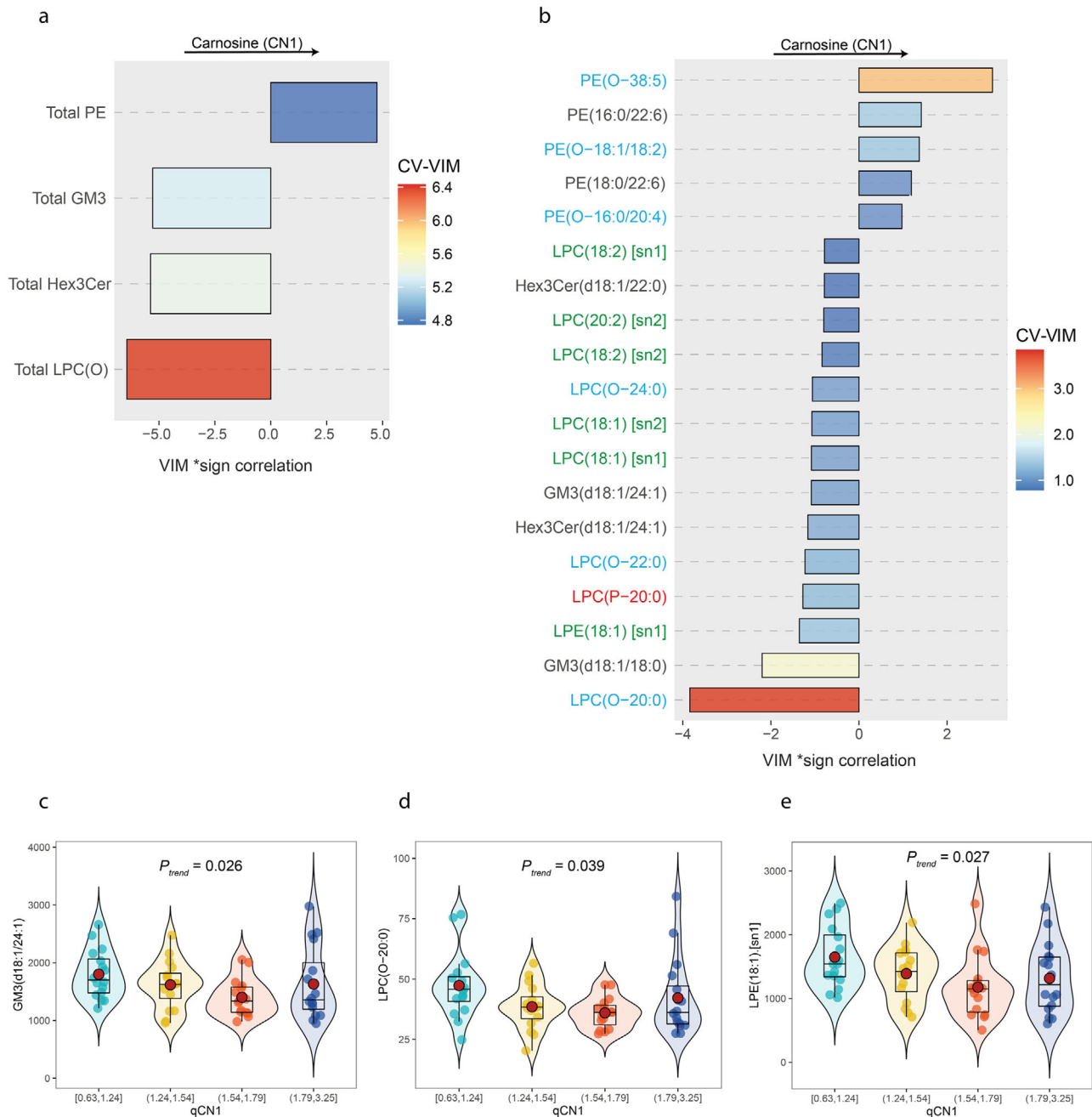


Figure 3. Associations of lipidomics profiles with plasma CN-1. **A)** Cross-validated permutation variable importance (CVPVI) measure \times sign of the correlation between each lipid class and **(B)** lipid species associated with the plasma CN-1. **C-E)** Associations of selected lipid species according to CN-1 quartiles

from disease status or the use of medications or supplements. In addition, we employed rigorous statistical multivariate and machine learning analyses.

In summary, muscle carnosine and serum CN-1 showed notable associations with the plasma lipidome that reinforce their possible role in the interactions between low-grade chronic inflammation, glucose homeostasis, and insulin sensitivity. Further studies are needed to confirm these findings and to establish whether manipulation of carnosine concentrations

using supplementation would influence lipid metabolism and subsequent metabolic disease risk.

4. Experimental Section

Study Population: The study population consisted of 65 overweight or obese (body mass index $> 25 \text{ kg m}^{-2}$) community-dwelling individuals from the Melbourne area (Australia) aged 18–57 years. The study protocol

was approved by the Monash University Human Research Ethics Committee (Protocol ID: CF13/3874-2013001988) and conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided written informed consent.

Based on medical history and laboratory and physical examination, participants were excluded if they had CVD, diabetes, respiratory, gastrointestinal, kidney, endocrine, hematological, or central nervous system diseases, as well as active cancer or cancer within the five preceding years. Exclusion criteria also included psychiatric disorders, acute inflammation, smoking, high alcohol use, and use of medications, vitamins, or supplements. Pregnant, lactating, or menopausal women were also excluded.

Anthropometric, Clinical, and Biochemical Measurements: Detailed methods for data collection and analysis have been previously described.^[35,36] Body weight (kg) and height (m) were measured and used to calculate the body mass index (BMI) (kg m^{-2}). Dual-energy X-ray absorptiometry was used to measure fat mass. Systolic (SBP) and diastolic blood pressure (DBP) were derived from three measurements using an automated sphygmomanometer (M6 Automatic BP monitor, Omron) after at least 5 min rest. A 2-h 75g oral glucose tolerance test (OGTT) was performed after a 12-h overnight fast to assess fasting, 1 and 2-h glucose as well as fasting insulin. Insulin sensitivity (M-value) was assessed by hyperinsulinemic-euglycemic clamps as previously described.^[35] Lipid profiles, including total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), were analyzed using commercial enzymatic immunoassays. Serum high sensitivity C-reactive protein (hsCRP) was measured by highly sensitive near-infrared particle immunoassays on Synchron LX analyzers (BC, Australia). Serum pro- and anti-inflammatory cytokines and adipokines were measured using commercial bead-based multi-analyte assays (LEGENDplex, Biolegend, CA, USA) and acquired on a BD LSR II flow cytometer as previously described.^[37]

Lipidomics: Targeted lipidomics analyses were performed as previously described.^[23] Detailed information can be found in the supplementary methods and supplementary Table 1 (Supporting Information). We were able to identify 459 lipid species across 26 different lipid classes and subclasses. These included major sphingolipid, phospholipid, glycerolipid, and sterol classes and the major species within each of these classes, which represent the major species present in circulation.

Muscle Carnosine and Carnosinase Measurements: Skeletal muscle tissue was obtained from the vastus lateralis by percutaneous muscle biopsy under local anesthetic. Samples were frozen immediately using liquid nitrogen and were stored at -80°C for later batch analysis.^[35] Skeletal muscle carnosine levels were quantified using reversed-phase high-performance liquid chromatography (HPLC). Skeletal muscle samples were dissolved in phosphate buffer (1mg ww muscle/15 μL PBS) for homogenization. Muscle homogenates were deproteinized using 35% sulfosalicylic acid (SSA) and centrifuged (5 min, 14 000 g). 100 μL of deproteinized supernatant was dried under vacuum (40°C). Dried residues were resolved with 40 μL of coupling reagent: methanol/ triethylamine/ H_2O / phenylisothiocyanate (PITC) (7/1/1/1) and allowed to react for 20 min at room temperature. The samples were dried again and resolved in 100 μL of sodium acetate buffer (10 mM, pH 6.4). The same method was applied to the standard solution of carnosine (Flamma). The derivatized samples (20 μL) were chromatographed on a Waters HPLC system with an ODS2 guard column (80 \AA , 5 μm , 4.6 mm X 10 mm), a Spherisorb C18/ODS2 column (4.6 \times 150 mm, 5 μm), and UV detector (wavelength: 254 nm). The columns were equilibrated with buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid) and buffer B (60% acetonitrile-40% buffer A) at a flow rate of 0.8 mL min^{-1} at 25°C .

Serum carnosinase concentrations (CN-1) were determined by a sandwich ELISA (enzyme-linked immunosorbent assay) developed by Adelman.^[38] High absorbent 96-well plates (Lab NUNC-Immuno Plate Maxi Sorp F96, Fisher Scientific GmbH) were incubated overnight with 100 μL of goat polyclonal anti-human CN-1 (10 $\mu\text{g mL}^{-1}$) (R&D, Wiesbaden Germany). Afterwards, the plates were extensively washed (200 μL Tween20 in 100 mL PBS) and incubated with a blocking buffer (0.05 % W/V of dry milk powder) for 40 min on a shaker (350 rpm), followed by

extensive washing. Next, 100 μL of sample (dilution: 1/300) and standard (recombinant human CN-1, R&D Systems; serial dilution) were added. The plates were placed on a shaker for 1 h and subsequently extensively washed. Thereafter, ATLAS anti-human CN-1 antibodies (Sigma PA) were added for 1 hour followed by extensive washing. Goat anti-rabbit IgG HRP (horseradish peroxidase) (Santa Cruz) was added for 30 min. Again, extensive washing was performed. By adding deep-blue peroxidase (POD) (Roche diagnostics) a color change was generated. This reaction was generally stopped after 10 min by addition of 50 μL of 1 M H_2SO_4 . The plates were directly read at 450 nm. CN-1 protein concentrations were assessed in the linear part of the dilution curve.

Statistical Analysis: Univariate statistical analysis was performed using version 23 of the Statistical Package for the Social Sciences (IBM SPSS Statistics) and R. Before analysis, data normality and homogeneity of variances were evaluated visually and using the Shapiro-Wilk and Levene's tests, respectively. Results are expressed as counts and frequencies (%) for categorical variables, means and standard deviations (SD) for normally distributed continuous variables and medians and interquartile ranges (IQR) for non-normally distributed continuous variables. Between-group differences were assessed using the χ^2 test for categorical variables, unpaired Student's *t* test for normal continuous variables, and Mann-Whitney *U* test for non-normal continuous variables. The associations of muscle carnosine and serum CN-1 with anthropometric, clinical, and biochemical variables were evaluated using partial Spearman correlations adjusting for age, sex, and fat mass. The relationships among quartiles of muscle carnosine and serum CN-1 with the other cardiometabolic risk factors were assessed using generalized linear models (GLM) with a gamma distribution and a logarithm link function to account for normality, adjusting for age, sex, and fat mass.

The identification of significant relevant features (lipid species or classes) associated with muscle carnosine and serum CN-1 levels was performed combining multivariate supervised projection methods and machine learning-based variables techniques. Before supervised analyses, principal component analyses were performed to identify strong outlying samples containing extreme lipid values. Then, supervised orthogonal partial least squares (O-PLS) regression models were built using in-house Matlab scripts to identify those lipidomic features that were associated with muscle carnosine and serum CN-1 levels. Here, the lipid species or classes were used as the descriptor matrix (X) and carnosine or CN-1 were used as response variables (Y). The models' performance and validity were calculated using a leave-one-out cross-validation approach and permutation testing (1000 permutations), respectively. Significant features were selected based on the O-PLS regression loadings adjusted for multiple testing using the Benjamini-Hochberg procedure for false discovery rate (FDR). A $p\text{FDR} < 0.05$ was used as the reference feature selection criterion. Usually, in high-dimensional omics datasets, many variables do not carry information and only a small set of variables is associated with the outcome. Adding all variables in the model can thus decrease the global model predictive performance. Therefore, we further analyzed the results adopting a random forest-based machine learning variable selection strategy based on the Variable Importance Testing Approach (vita) algorithm as implemented in the *vita* R package.^[39] In addition to performing variable selection, tree-based methods can capture complex nonlinear and complex interactions between the predictor variables, which are common in omics datasets. The variable importance measure (VIM) in the *vita* algorithm is based on cross-validated permutations. The method randomly splits the data in *k*-folds of equal size. For each *i*-fold, a random forest is trained using all samples that are not part of the *i*-test set, and the response variable is predicted for the samples in the *i*-test set. The procedure is repeated after permutating *n* times the values of the predictor variables. The VIM is calculated as the average difference in the prediction errors between the original data and permutations, and the CV-VIM is the average overall *k*-fold-specific permutation VIM. For non-relevant features, the change in accuracy is only due to random variations and thus it does not change (zero VIM) or slightly increases (negative VIM) when not using the variable for prediction in the random forest. *p*-values are then calculated approximating the null distribution of VIM scores

based on the observed non-positive scores of non-relevant variables. *p*-values were then corrected using the Benjamini-Hochberg procedure for FDR.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.-M.F.-R. and B.d.C. are senior authors. J.M.P. performed data analysis and interpretation and wrote the first draft of the manuscript. W.D. performed the muscle carnosine analysis and contributed to writing and editing the manuscript. A.M. and N.N. performed data collection and contributed to writing and editing the manuscript. P.M. supervised the lipidomics laboratory analysis and contributed to writing and editing the manuscript. J.-M.F.-R. supervised the data analysis and contributed to writing and editing the manuscript. B.d.C. conceptualized this study, is the chief investigator of the original study, obtained funding, oversaw data collection and analysis, and contributed to data interpretation and writing and editing the manuscript. J.-M.F.-R. and B.d.C. are the guarantors of this work and takes responsibility for data integrity and accuracy. All authors meet the ICMJE criteria for authorship and have approved the final version of the manuscript for publication.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

carnosine, iron, lipidomics, muscle, obesity

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